Exploring the potential of Berberine for its Anticancer Immunity

A thesis submitted to the

Institute of Advanced Research

for the award of degree

of

Doctor of Philosophy

By

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July, 2023

Thesis Abstract: Exploring the potential of Berberine for its Anticancer Immunity

Macrophages are sentinel cells that are important for immune surveillance. Tumor-immune cell interaction redirects macrophages to an immunosuppressive phenotype, that aids in tumor progression. Restoring tumor-associated macrophages (TAMs) to antitumor phenotype at the Tumor-Immune Micro-environment (TIME) may facilitate better immune outcome. In the presented work, we have investigated the potential of berberine in macrophage polarization in TIME and subsequent T cell activation. We have observed that berberine enhanced tumor antigen-induced IL-1β, IL-12 and TNFα, but suppressed IL-6 and TGFβ. It rescued IL-10-induced immunosuppressive macrophages to release pro-inflammatory cytokines. Since TIME consists of tumor derived soluble factors and tumor antigens, so in order to generate TIME for *in vitro* conditions, we have used the culture supernatant from confluent B16F10 cells along with crude soluble antigen forming the Conditioned Media (CM). CM-induced BMDMs skewed to an immunosuppressive phenotype as evidenced by increased CD206, but decreased MHC-II expression. It was also confirmed by decreased arginase-1 activity and mitigated levels of IL-10, IL-6 and TGF-β. It improved antigen specific T cell response by enhancing MHC-II, CD40 and CD86 expression that augmented the CTL activity and IFNγ-producing $CD4^+$ T_H-1 subset. In melanoma tumor model, berberine significantly enhanced the frequency of M_1 macrophages. Berberine treatment decreased $CD4^+$ $FOXP3^+$ regulatory T cells population and enhanced $IFN\gamma^+$ $CD8^+$ T cells in tumor-bearing mice. We observed that berberine, via STAT-3 inhibition, prevented the generation of TAMs, which was evident by decreased *CEBP/β* and *Egr2* mRNA expression; and reduced IL-10 and TGFβ production. STAT-3 promotes arginase-1 expression that plays a crucial role in L-arginine depletion in TIME. Berberine decreased arginase-1 expression and subsequently its activity as evident by reduced urea release during L-arginine supplementation, confirming our finding of berberine mediated STAT-3-arginase-1inhibition. Later, to enhance the immunomodulatory potential of berberine, we have used a combination of L-norvaline (arginase-1 inhibitor) and berberine. The combination treatment significantly reduced tumor growth by enhancing $CD4^+$ T_H-1 subset, $IFN\gamma^+$ $CD8^+$ T cells with improved CTL activity and decreased $FOXP3^+$ T_{reg} cells. Hence, the presented work indicates that berberine prevents immune tolerance by skewing macrophages to anti-tumor phenotype via STAT-3-arginase-1 inhibition. The combination treatment of berberine and L-norvaline significantly improved antitumor immune response with reduced tumor growth, suggesting a better therapeutic approach for targeting TIME

Key words: Macrophage polarization, TIME, Berberine, L-arginine metabolism

Introduction:

The project is focused on understanding the immunomodulatory properties of berberine in the tumor microenvironment. Cancer is probably one of the most studied diseases in Humans. By decades of extensive research, it turns out to be characterized by genetic and epigenetic instability leading to the generation of unique, multiple and uncommon mutations. These alterations promote the elicitation and expansion of the adaptive immune system that functions predominantly to distinguish between cells bearing cancerous phenotype from normal cells. Thus, the immune system of a patient with cancer develops an intrinsic capability of recognizing his/her own cancer however, the degree in each individual may vary greatly.

In 1957, F.M Brunet and L. Thomas first formulated the concept of immune surveillance. Later on, with the advancement of knowledge in the field of immunology, the concept of immune tolerance came into existence. This finding laid the milestone for development of a new hypothesis termed as 'cancer immunoediting'. In 2004, Dunn et al have suggested three phases of cancer immunity cycle: Elimination, Equilibrium and Escape. Elimination: Abnormally transformed cells are potentially harmful to the host. The immune system recognizes and eliminates these cells. Equilibrium: This phase continues to a point where tumor cells establish equilibrium and the theoretically the tumor cell proliferation is equal to the cell death caused by the immune system. Escape: In this phase, the edited tumor cells are no longer restricted by the host immunity and develop aggressively. This phase laid the foundations of the tumor microenvironment. The tumor microenvironment is highly dynamic as it harbor many cells types including nascent and senescent neoplastic cells, stromal cells, endothelial cells, immune cells, fibroblasts, cancer stem cells and many more. The bidirectional interaction between the cells in the tumor microenvironment is mediated by cytokines, chemokines and growth factors. These interactions alter the immune response from anti-tumor to pro-tumor responses.

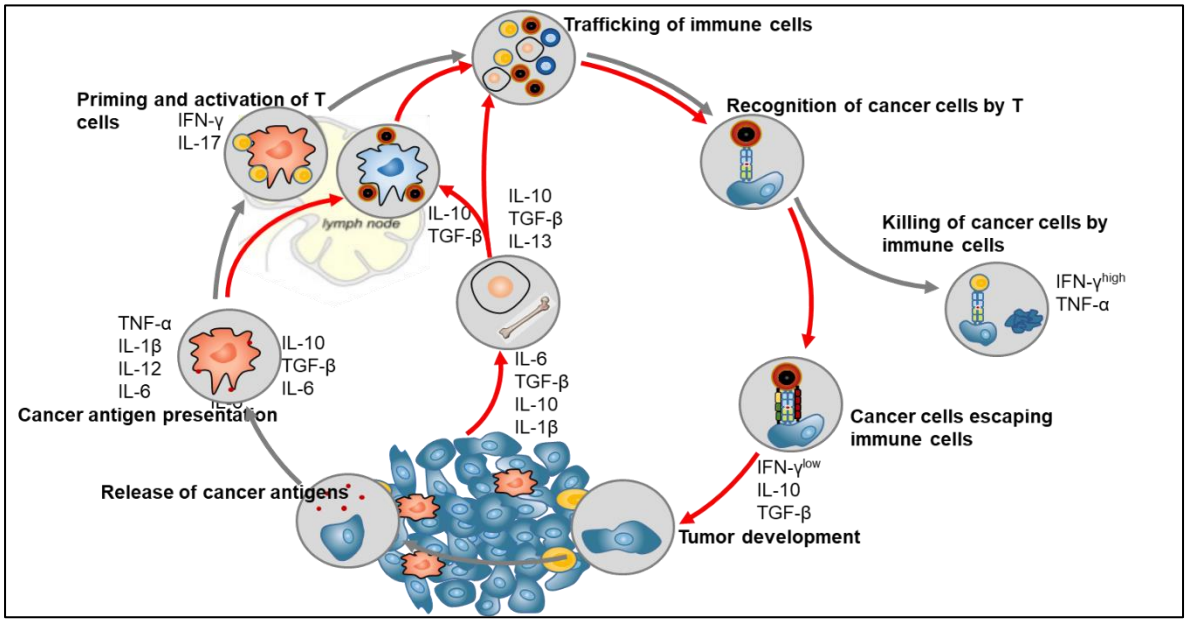


Fig. 1 Cancer-Immunity Cycle

Macrophages are broadly classified into two distinct phenotypes: classically-activated anti-tumor or M1-like and alternatively-activated pro-tumor M2-like macrophages. While the former macrophage subset releases pro-inflammatory cytokines and promotes TH1 and cytotoxic T-cell (TC) response, M2-like macrophages release anti-inflammatory cytokines such as IL-10 and TGF-β. TAMs are M2-like macrophages that promote tumor progression through various mechanisms such as tumor cell proliferation by secreting various growth factors, angiogenesis by inducing VEGF, metastasis by secreting matrix metalloproteinases, and preparing an immunosuppressive environment by releasing

IL-10 and TGF-β in the tumor milieu. While IL-10 promotes monocytes differentiation to M2-like macrophages, TGF-β induces regulatory T (T-reg) cells or, in presence of IL-6, TH17 cells. TH17 cells were shown to play both pro-tumor and anti-tumor roles in mice. All these processes contribute to enhanced generation and distribution of TAMs ensuring the maintenance of immunosuppressive microenvironment. In addition, tumor cells dampen effector T-cell response leading to T-cell anergy. Pathological studies have shown that low survival rate is associated with the presence of TAMs in neuroblastoma. Recent studies suggest that blocking TAMs/M2 macrophage polarization and functions or reverting to M1-like phenotype is an attractive principle for anti-cancer therapy. How M2-like macrophages are converted to M1-like macrophages remains unknown.

In presence of tumor-derived factors, macrophages undergo functional polarization to generate tumor-associated macrophages (TAMs) that are anti-inflammatory or alternatively-activated and help in tumor progression, metastasis and immune suppression. For a very long time, cancer therapy relies on targeting cancer cells but these therapies often fail due to incomplete removal of the cancer cells (e.g., in surgery), of resistance of cancer cells to drugs (e.g., in chemotherapy) or escape of cancer cells from death (e.g., relatively radio-resistant cancer stem cells after radiotherapy). Therefore, immunotherapy holds much promise as it targets host immune system to turn it efficient against the tumor cells leading to tumor regression. One of these immunotherapeutic targets is equally essential to surge for therapies that target macrophages.

Italian oncologist Alberto Mantovani laid down the foundation of macrophage polarization correlating inflammation and tumorigenesis. The macrophages are of two phenotypes: Classical macrophages (M1) and Alternative macrophages (M2). In activated macrophages (M1), there is upregulation of pro-inflammatory cytokines resulting into cancer cell apoptosis. But in the immunosuppressive milieu, they are polarized to tumor-associated macrophages (M2-phenotype or alternatively activated). TAMs promotes tumor progression by enhancing angiogenesis, metastasis, and immune evasion. The difference in polarized macrophages is based on the L-arginine metabolism. The two key enzymes iNOS and Arginase-1 metabolize L-arginine to different products. In classically activated macrophages, iNOS metabolize L-arginine to nitric oxide and citrulline which induces toxicity via reactive nitrogen species while in alternatively activated macrophages, Arginase-1 metabolizes L-arginine to ornithine and urea which later promotes cancer cell proliferation and survival. Thus, L-arginine is metabolized to different products depending on the macrophage phenotype.

Transcription regulation of iNOS and Arginase-1: Various transcription factors play an important role in upregulation of iNOS gene. LPS mediated activation of NFκB binds to the promoter region of iNOS. IFNs binds to STAT-1α or NFκB mediated activation of STAT-1 causes activation of iNOS. Hypoxia response element causes upregulation of iNOS.

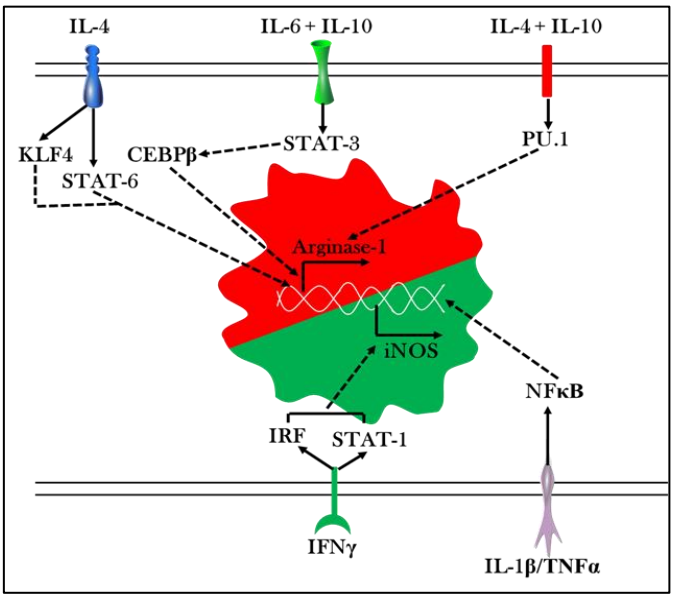


Fig. 2 Transcriptional regulation of Arginase-1 and iNOS

Various cytokines such as IL-6, IL-4 and IL-10 activates Arginase-1 by STAT-3/6 and PU.1. In addition to this, STAT-6 co-operates with KLF4 to induce Arginase-1 activation. Subsequent studies have showed TLR-dependent activation of C/EBPβ induces secretion of cytokines which in turn activates STAT-3 which upregulates Arginase-1. Recently, increasing reports have indicated PPARs facilitates Arg-1 transcription. Thus, various cytokines/growth factors/other factor affects the transcription regulation of Arginase-1 and iNOS.

M1 macrophages drives naïve CD4+ T cells towards Th1 subset may further promote tumor rejection Th1 cells promote activation of CD8+ cytotoxic T cells. On the contrary, tumor progression is associated with polarization toward a Th2 / regulatory T cell response which is induced by M2 macrophages. This cells exert immune suppressive functions through release of immunosuppressive cytokines (IL-10, TGFβ) and enhanced expression of co-inhibitory molecules leading to dysfunctional T cells. They inhibit recognition and clearance of tumor cells by the immune system. Thus, cancer cells modulate the immune cells in order to maintain its existence. These polarized macrophages compete for substrate availability. In TME, L-arginine is consumed by Arginase-1 which is highly expressed in melanoma cells and TAMs compared to iNOS. Thus, there occurs depletion in L-arginine availability to the immune cells present in the microenvironment which causes T cell inactivation

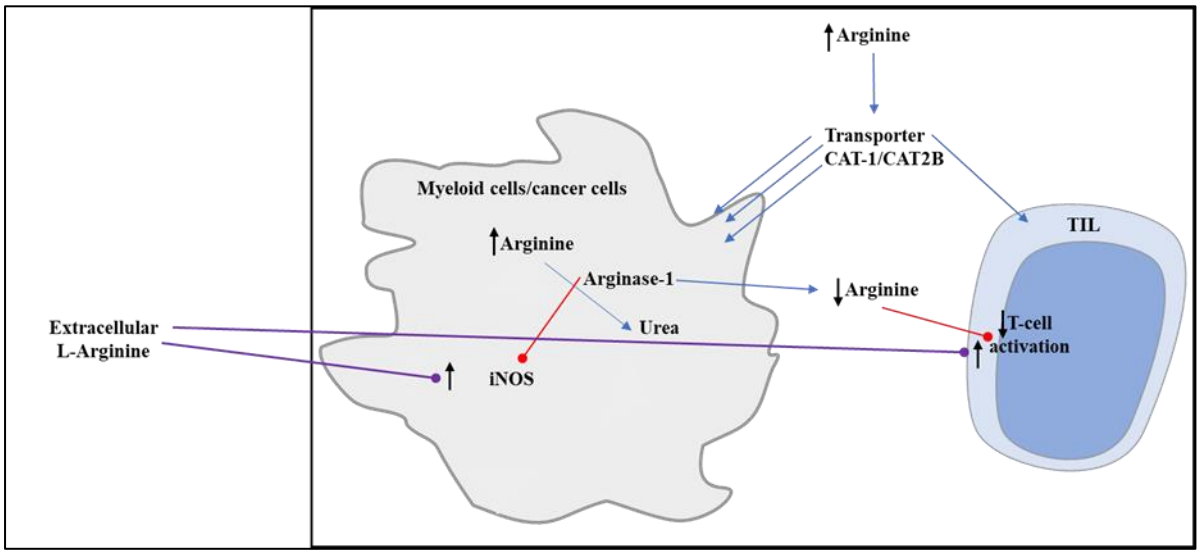


Fig.3 Impact of L-arginine in TME

Recently the major area of research in tumor immunology has been directed on targeting the multiple cell type and molecular interactions with the tumor microenvironment. This has led us to the new era of therapeutics such as immunotherapy, monoclonal antibody based therapy, CAR T cells etc. But due to limitations like immune toxicities, target specificities, high cost, disparity in patient to patient response etc the search for an effective therapy still continues. Phytomedicine is widely used as a therapeutics since ages. Recently, the research interest is focused on understand their potential in immunomodulation. Berberine is an isoquinoline alkaloid isolated from various medicinal herbs such as Coptis chinensis, Berberis vulgaris, Berberis aquifolium, Berberis asiatica, etc. It was traditionally used as a medicine to treat diarrhoea, infections and metabolic disorders, etc. We have previously reported anti-cancer potential of berberine via inhibition of migration, EMT reversal and decreased cancer cell stemness in neuroblastoma. Recent studies have shown that berberine selectively modulates different pathways depending on the cell type. Berberine is also shown to have immuno-modulatory effects. Hu Z et al, 2011 have shown that berberine selectively induced apoptosis of dendritic cells

suggesting its potential as a therapeutic agent for autoimmune disorders14. In the present study, we aimed at studying the effect of berberine on macrophage polarization in the TME and its potential in restoring skewed macrophages towards inflammatory M1 phenotype.

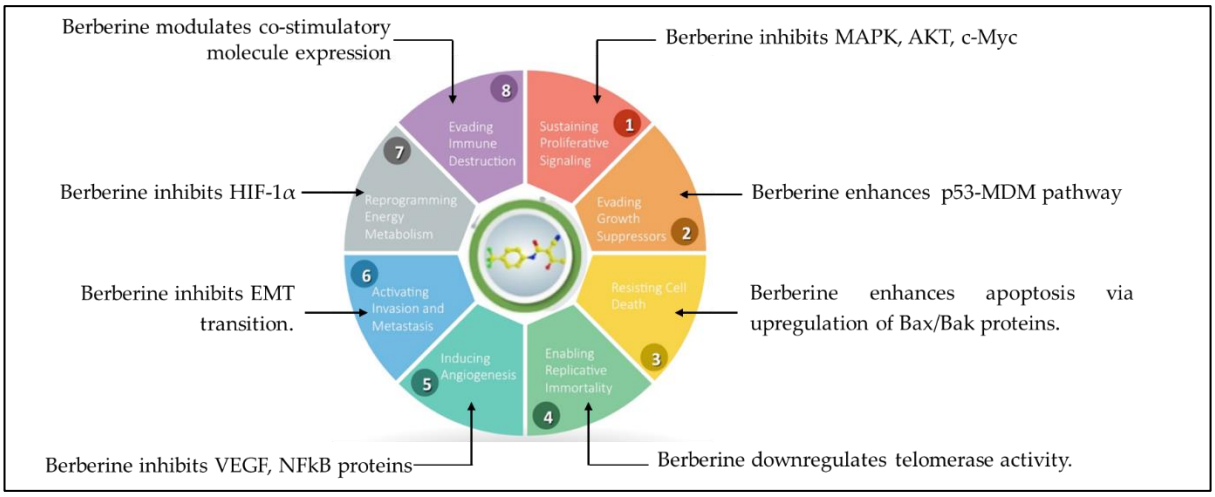
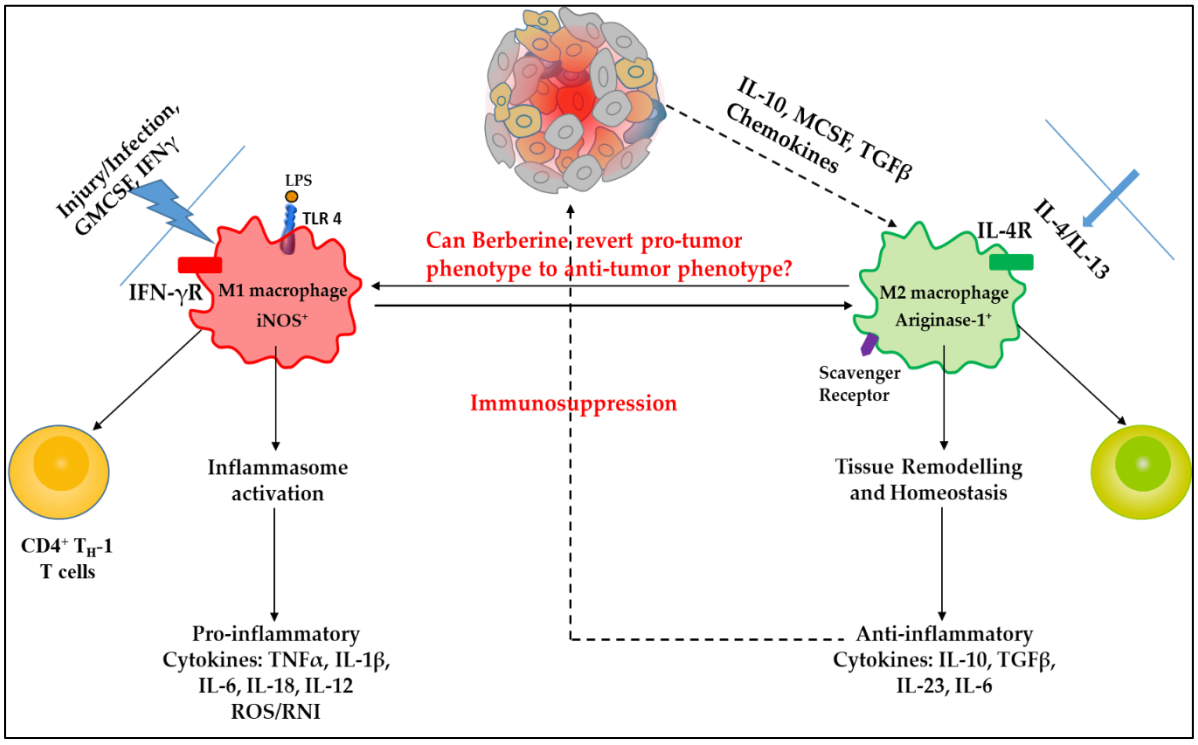


Fig.4 Anticancer potential of Berberine

Hypothesis:

Cancer cells secrets high concentration of immunosuppressive cytokines as in IL-10, IL-6 and TGFβ via STAT-3 activation. These cytokines skews macrophages to alternative phenotype-TAMs which in turn releases immunosuppressive cytokines and maintains immune suppression in the tumor microenvironment. These cytokines inhibit pro-inflammatory cytokines and also aids in recruitment of regulatory T cells which dampens the cytotoxic T cell response. So, we wanted to check the effect on berberine in manipulating tumor microenvironment. Hence, we hypothesize that berberine may help in restoration of classical macrophages via inhibition of STAT-3 pathway



Objective:

- To test the effect of berberine on innate immune cells and their anti-cancer functions
- To test the effect of berberine on STAT-3 regulation under differential conditions
- Testing the effect of berberine on tumor specific Antigen Presenting Cells and T cell interaction via MHC-II, CD40/CD40L and PD-1/PD-L1
- Testing the efficacy of berberine in TME *in vivo*

Materials and Method:

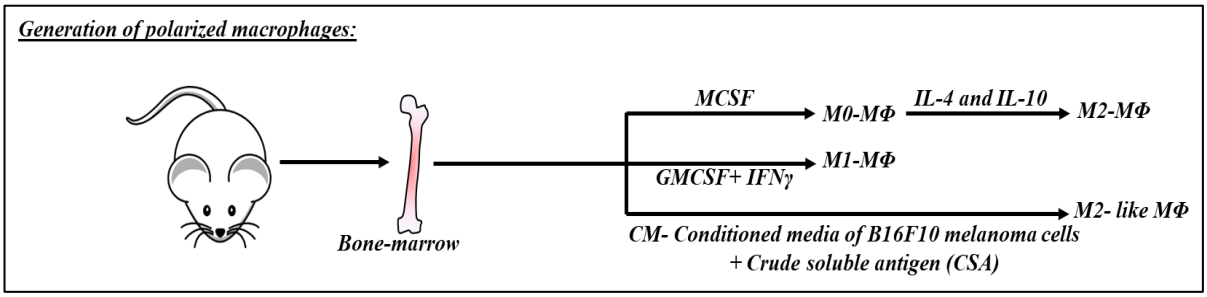
Cell lines: Raw264.7-mouse macrophage cell line
B16-F10- mouse melanoma cell line

Preparation of conditioned media and cancer cell lysate:

Crude soluble antigen/tumor antigen (CSA) was prepared using freeze-thaw methodology. Confluent B16-F10 cells were freeze-thawed for 7 repetitive cycles of -80°C/37°C for 10 minutes each and centrifuged at 10,000 rpm for 15 minutes. The protein concentration was quantified using Bradford assay. The culture supernatant was harvested from 95% confluent B16-F10 cells maintained in DMEM containing 2% FBS in last 24 hrs. To depict the tumor mimicking conditions, conditioned media (CM) was prepared by mixing 10µg mL^{-1} of CSA to 200µl of culture supernatant. These concentrations were maintained throughout the study and the CM was used with fresh media at 50/50 V/V to ensure nutritional availability.

Preparation of bone-marrow derived macrophages (BMDMs):

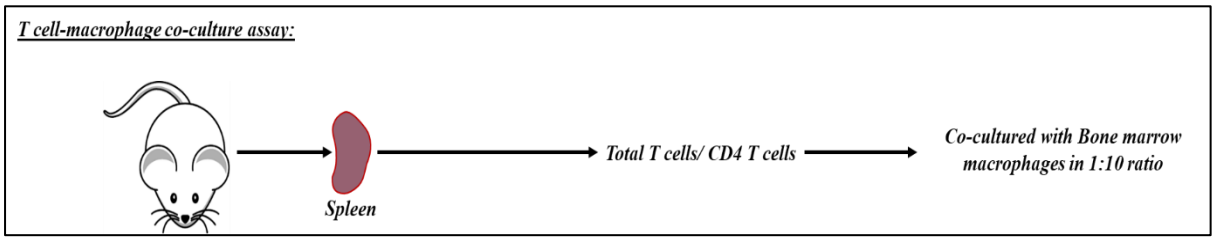
BMDMs were prepared by isolating bone-marrow cells from femur and tibia of BALB/c mice. The cells were washed and re-suspended at 10^6 cells mL^{-1} in 10% RPMI 1640 containing MCSF (20ng mL^{-1}) for macrophage generation (M0). For polarization, the cocktail of GM-CSF (20ng mL^{-1}) and IFNγ (10ng mL^{-1}) was used for M1 while MCSF (20ng mL^{-1}), IL-4 (10ng mL^{-1}) and IL-10 (10ng mL^{-1}) for M2 macrophages. At day 6, the BMDMs were 95% CD11b positive (not shown) and were used for the experiments. The toxicity of berberine on polarized macrophages was checked.



T-cell purification and Macrophage (M0)-T cell co-culture:

$CD4^+$ T-cells were isolated from spleen of BALB/c mice using $CD4^+$ T-cell enrichment kit. The BMDM-CD4+ T-cell co-culture assay was performed as described. The macrophages (M0) were treated with CM in the absence/presence of berberine (10µM mL^{-1}) for 24 hr. $CD4^+$ T-cells were added to the culture at a ratio of 1:10. For mRNA analysis, the co-cultured cells were incubated for 12 hr. and the

non-adherent T-cells were used for RNA isolation and mRNA expression. For scoring cytokines, the co-cultures were incubated for 72 hr and the supernatant was used for ELISA.


T cell-macrophage co-culture assay:
Spleen
Total T cells/ CD4 T cells
Co-cultured with Bone marrow macrophages in 1:10 ratio

Semi-quantitative RT PCR and Real-time PCR:

Gene expression was assayed by semi-quantitative RT-PCR as described previously [16]. Briefly, the total RNA was extracted and quantified. 2µg of RNA was used for cDNA synthesis by iScript cDNA synthesis kit (Bio-Rad, USA). The cDNA was amplified with gene-specific primers (Supplementary table 1a) using the following conditions: 95°C for 5 min, 95°C for 30 s, 55–60°C for 45 s, 72°C for 1 min for 25–30 cycles, followed by a final extension at 72°C for 10 min. β-Actin was amplified from each sample to ensure equal cDNA input. For real-time PCR, 100ng of cDNA was used for amplification of mentioned genes in triplicates using gene-specific primers (Supplementary table 1b) using Power SYBR green 2X Master Mix in Applied Biosystems Step One Plus Thermal cycler and analysed with SDS2.4 software (Applied Biosystems).

Blue native-poly acrylamide gel electrophoresis (BN-PAGE):

STAT3 dimerization was analysed by shift in protein bands by modifying previously established BN-PAGE. Protein was extracted from rIL-6 stimulated macrophages in presence/absence of berberine in blue native gel solubilisation buffer (50mM Sodium chloride, 50mM Imidazole, 1mM EDTA, pH adjusted to 7.0 with HCl). 100µg of protein was separated in BN-PAGE ((20%, 15%,12% (pH 8.8)), (10%, 8%, 6% (pH 7.2)), 4% (pH 6.8)) in presence of cathode (50mM Tricine, 7.5mM Imidazole, 0.002% Coomassie blue G-250, pH 7.0) and anode buffer (25mM Imidazole, pH adjusted to 7.0 with HCl). Resolved protein in BN-PAGE was used for western blotting as mentioned in the following to observe total STAT3 monomers and dimers.

Western Blotting:

Protein expression was assayed by Western blotting as describe. Briefly, 50µg of protein was resolved on a SDS-PAGE gel and electro-blotted onto PVDF membrane. The membrane was blocked with 5% non-fat milk or 2% BSA in tris-buffered saline for 2 h at room temperature. The membranes were probed overnight at 4°C with the following primary antibodies: sheep anti-mouse Arginase-1, rabbit anti-mouse iNOS, anti-mouse pSTAT-3, T-STAT-3 and GAPDH at 1:1000 dilutions. Later, the membranes were probed with HRP-conjugated secondary antibody (1:5000) for 1 h at room temperature followed by detection with ECL kit.

U.V spectral analysis of STAT-3-bound protein:

STAT3 was immune-precipitated from macrophages stimulated with IL-6 in presence/absence of IL-6 using STAT-3 antibody which was later pooled by protein agarose-A/G. The obtained STAT3 was washed thrice with PBS and UV-visible spectra were measured at room temperature on a Shimadzu

1900i spectrophotometer equipped with 0.5 mL quartz cell. Berberine concentration and protein concentration was obtained from standard graphs plotted at 340nm and 280nm respectively. STAT-3 bound Berberine was calculated as nM of Berberine/µg of protein.

ELISA:

The BMDM cells were seeded at a density of 10^5 cells/well in triplicates with indicated treatments and the cell free supernatant was used for scoring cytokines by ELISA. In some experiments, the macrophages- T cell co-culture supernatant were used to score T-cell cytokines.

Flow cytometry:

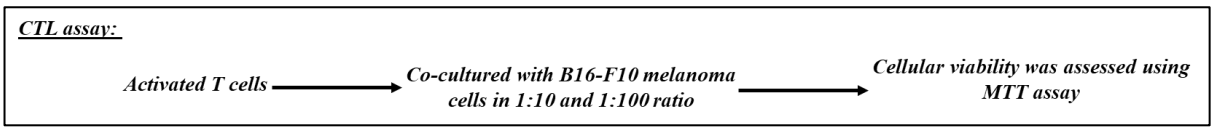
The BMDM were treated with TMC for 24 h followed by berberine (10µM mL^{-1}) treatment for 24 h and protein expression was analysed by flow cytometry as described. The cells were analysed for expression of: MHC-II (anti-rat FITC I-Ad), anti-goat CD206 (R&D Systems, NY, USA), anti-mouse CD40 (B.D. Biosciences, CA, USA), anti-mouse APC-Cy7 F4/80 (Biolegend, CA, USA) and anti-mouse iNOS-PE (B.D. Biosciences, CA, USA). Flow cytometry was performed using a FACS Canto II and analysis was done using CellQuest software (BD Bioscience, CA, USA) or Flowjo software.

Immuno-histochemistry and confocal microscopy:

Tumour tissue collected in 4% PFA and embedded in paraffin were cut into 4 micron thin sections and transferred to poly-lysine coated slides. Immuno-histochemistry for CD68, CD206, CD11b was done as described earlier. DAPI was used as nuclear stain. Appropriate flourochrome labelled secondary antibodies were used. Tissue sections were observed under confocal microscope (Leica TCS SP8). Images were analysed using LAS X 3.5.6.21594, Leica-Microsystems and Image J, NIH. Fluorescence area of each image was analysed to determine the cell population and plotted as relative fluorescence area.

Cytotoxic T-cell assay:

For CTL assay, total T-cells were purified from splenocytes using nylon wool column. Total T-cells were co-cultured with polarized macrophages as mentioned earlier. Later the primed T-cells, harvested from each experimental group were plated with N2a at ratios of 1:1, 1:10 and 1:100 for 5 days. The cytolytic activity of T-cells against B16F10 cells were analysed by MTT assay. Naïve T-cells activated with anti-CD3 and anti-CD28 antibody were used as control.


CTL assay:
Activated T cells
Co-cultured with B16-F10 melanoma cells in 1:10 and 1:100 ratio
Cellular viability was assessed using MTT assay

***In silico* studies:**

The molecular docking studies was carried out between pSTAT-3 (PDB id: 1BG1) and berberine (PDB id: 3NP6). The activation of STAT-3 is induced by phosphorylation of tyrosine residue (Y705) which then binds to the Src Homology 2 (SH2) domain of the other STAT-3 protein forming a homo-dimer. These STAT-3 homo-dimers translocate to the nucleus and regulate the transcription of various genes. The pTyr of STAT-3 interacts with Lys591, Arg609, Ser611, Glu612 and Ser613 of the SH2 domain.

Based on these, we have selected the active site that includes residues Lys591, Arg595, Arg609, Ser611, Glu612, Ser613, Ser636, Pro639, Tyr640 and Gln644. A grid box was generated around this site and molecular docking was carried out using AutoDock 4.2. UCSF Chimera and Ligplot programs were used for visualization, and analysis of ligand-protein interactions.

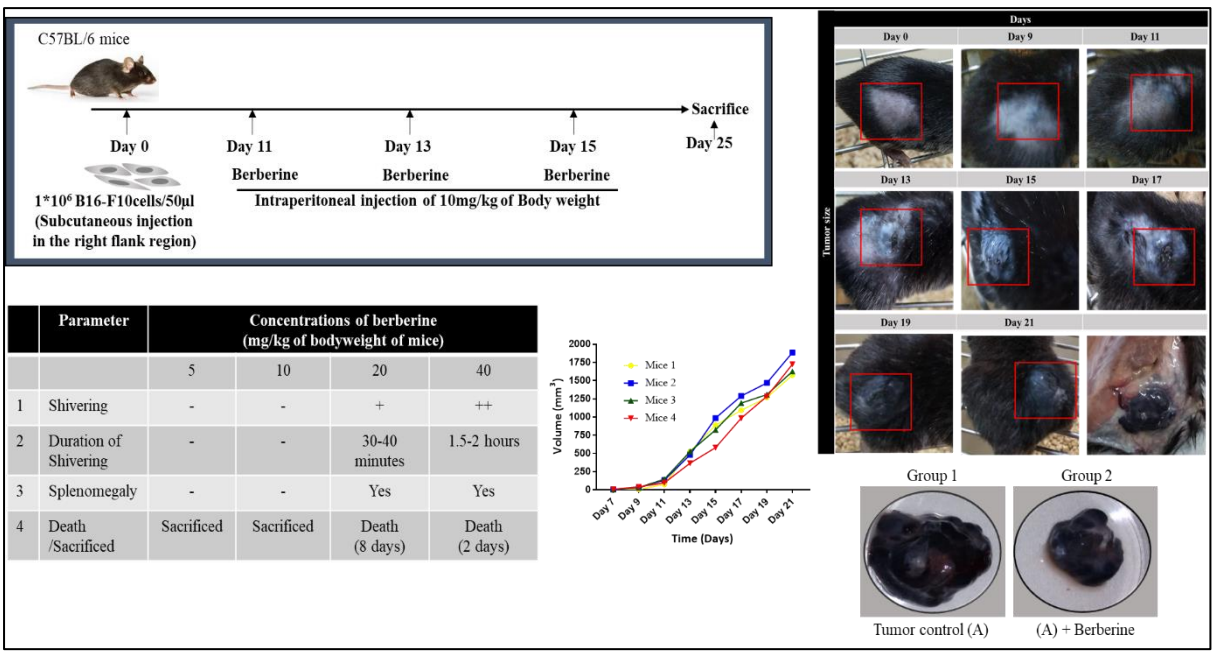
String analysis was done with proteins and mRNA levels analysed in the study along with the known macrophage polarisation markers to elucidate the plausible pathway manipulation by berberine and the mechanism responsible for alteration in macrophage phenotype43. The network obtained from the String v11 was analysed by network analyser in the cytoscape 3.7.2. Size of the node denotes the number of the interaction with the other nodes in the network. Thickness of the line between nodes denotes interaction strength score.

Arginase-1 activity:

Protein were extracted and quantified using Bradford reagent They were normalized with 25mM Tris-Cl making total concentration of protein to 50µg 100µL^{-1}. Add 10µL of MnCl2 in each sample. Activation of enzyme was done by heating the sample for 10 minutes at 56°C. Incubate the activated enzyme with 0.5M L-arginine (100µL) at 37°C for 1 hour. After incubation, the reaction was stopped using stop solution 98% H2S04: 85% H3PO4: H20 (1:3:7). Later, 25µL of isonitrosopropiophene was added and incubated for 30 minutes at 95ºC. O.D. was measured at 540 nm.

***In vivo* syngeneic mouse melanoma model:**

For melanoma model of tumor, 3*10^5 B16-F10 cells were injected subcutaneously into the right flank of C57BL/6 mice (n=6) in 50µL sterile phosphate-buffered saline (PBS). Berberine was injected intraperitoneally with concentration of 10mg kg^{-1} body weight in 50µL of DMSO on days 11, 13 and 15 after the tumor was palpable. The tumor size was measured every alternate day using Vernier callipers, and the volume of the tumor was calculated using the formula (length*width*width)/2. The mice were sacrificed on day 25 of tumor inoculation and were weighed.



	Parameter	Concentrations of berberine (mg/kg of bodyweight of mice)			
		5	10	20	40
1	Shivering	-	-	+	++
2	Duration of Shivering	-	-	30-40 minutes	1.5-2 hours
3	Splenomegaly	-	-	Yes	Yes
4	Death /Sacrificed	Sacrificed	Sacrificed	Death (8 days)	Death (2 days)

Results:

Objective 1: To test the effect of Berberine on innate immune cells and their anticancer function.

1. Generation of Polarized macrophages:

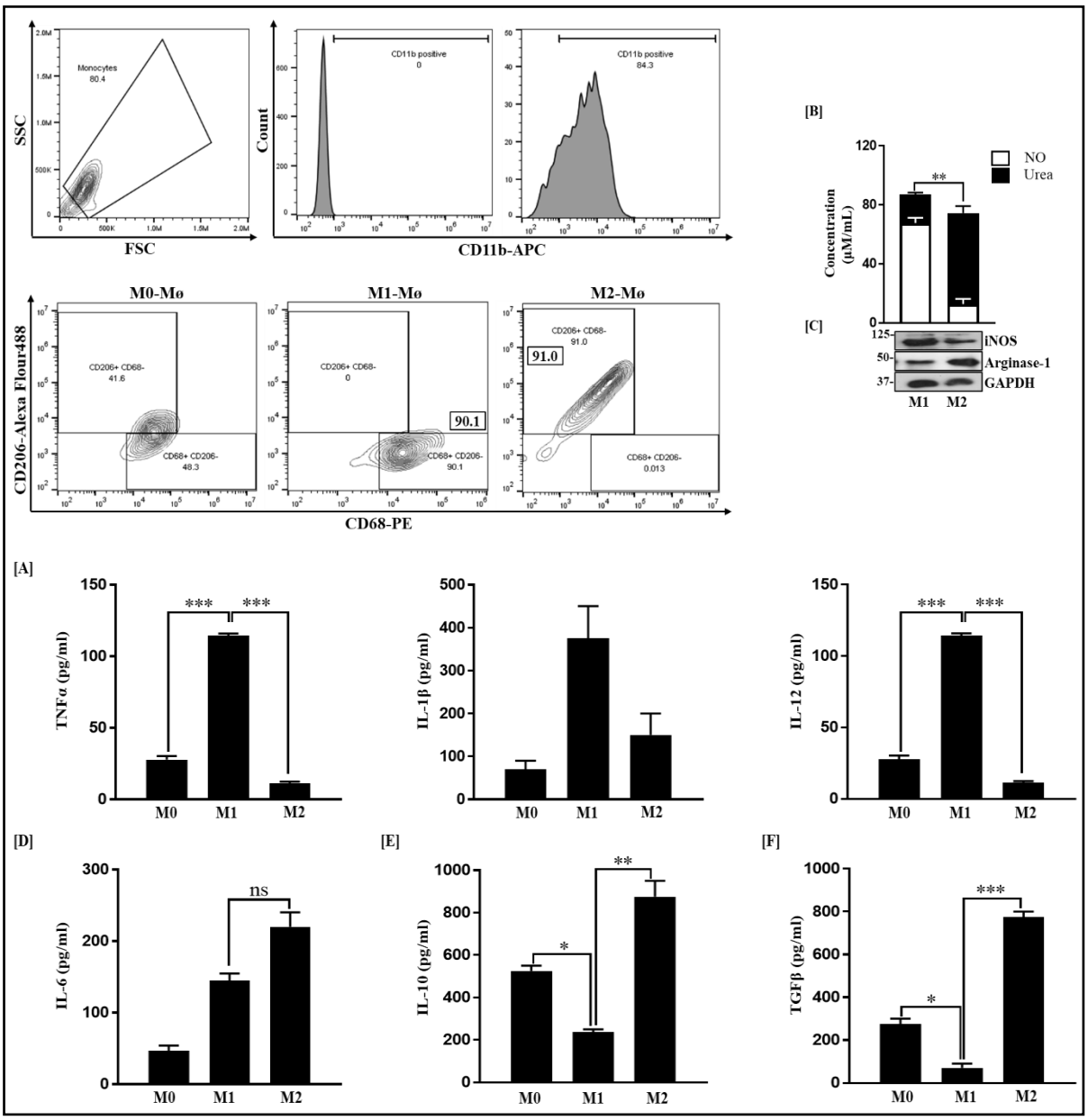
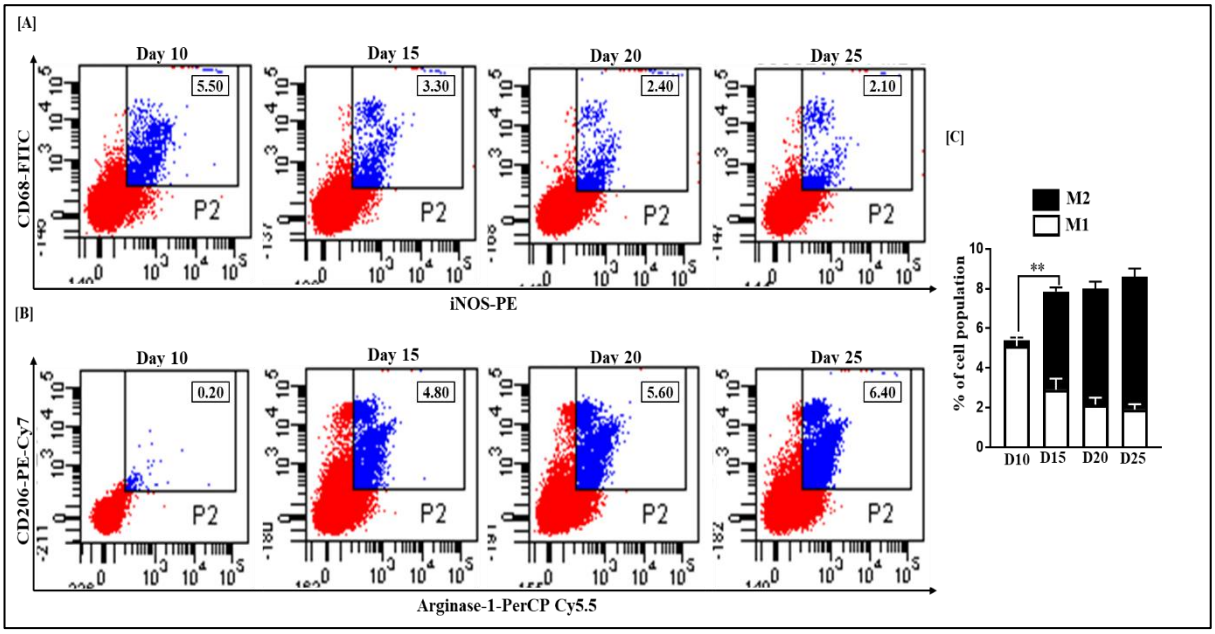


*Fig.1 BMDM (M0) were treated with cocktail of GMCSF (10ng/ml) and IFNγ (20ng/ml) for M1 polarized macrophages and MCSF (20ng/ml), IL-4 and IL-10 for M2 polarized macrophages for 7 days. The cells were then analysed for CD11b, CD68 (M1 marker) and CD206 (M2 marker) by flow cytometry The gated CD11b positive cells were further gated to CD68 and CD206 positive cells respectively [A]. The BMDM cells were treated with the M1 and M2 cocktail and checked for NO release by Griess method and urea by Arginase-1 activity [B], iNOS and Arginase-1 expression by western blotting [C]. BMDM (M0) were treated with cocktail of GMCSF (10ng/ml) and IFNγ (20ng/ml) for M1 polarized macrophages and MCSF (20ng/ml), IL-4 and IL-10 for M2 polarized macrophages for 7 days. The supernatant was collected and checked for various pro-inflammatory and anti-inflammatory cytokines by ELISA [A-F]. Data are of three experiments and represented as mean ± SEM. Statistical significance was determined by One-way ANOVA with Turkey's multiple comparison (**p < 0.001)*

In tumor-immune microenvironment, macrophage polarization is tumor-immune microne of the key mechanism BMDM were polarised to M1 and M2 macrophages and checked for M1-CD68 and M2-CD206 surface marker. We have observed that in M1 macrophages show high CD68 and iNOS

expression while M2 macrophages show high CD206 and Arginase-1 expression. This indicates that generated macrophage have polarised to M1 and M2 macrophages. The functional output was analysed by scoring various cytokines using ELISA.

Increased frequency of M2 polarised macrophages was observed in tumor tissue in time dependent:



*PFig.2 The tumor tissue were excised from the tumor bearing mice at different time points and analysed for M1 and M2 polarised macrophages by Flow cytometry. The tumor tissues were minced and stained for CD11b-APC, CD68-FITC, iNOS-PE (M1) and CD206-PE Cy7, Arginase-1 PerCP Cy5.5 (M2) and analysed by flow cytometry [A and B]. The % of cell population obtained is plotted [C]. Data are of three experiments and represented as mean ± SEM. Statistical significance was determined by Two-way ANOVA with Turkey's multiple comparison (**p < 0.001).*

It is reported that with increase in tumor growth, the macrophages are polarized to immunosuppressive phenotype in the tumor microenvironment. To confirm it, the tumor bearing mice were sacrificed at different time-points. We have observed that with the increase in the time points, there was increase in frequency of M2 macrophages in tumor micronvironment. This data is in accordance with our hypothesis.

Berberine improves the release of pro-inflammatory cytokines from macrophages:

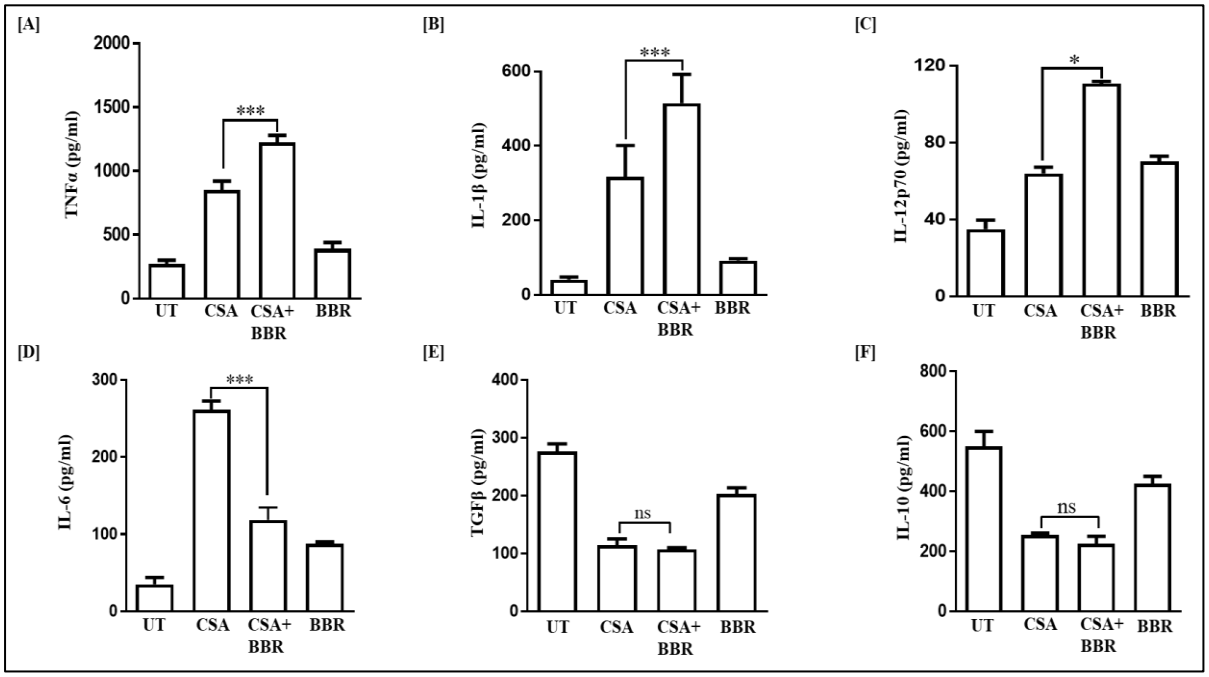


*Fig.1: Raw264.7 (murine mouse macrophages) were pre-treated with crude soluble antigen (CSA) for 15 hrs followed by berberine (10µg/ml) treatment for 24 hrs. The supernatant was collected to analyse various pro/anti-inflammatory cytokines by ELISA [A-F]. Data are of three experiments and represented as mean ± SEM. Statistical significance was determined by One-way ANOVA with Turkey's multiple comparison (***p < 0.0001; *p < 0.05; ns= non significant).*

In order to check the effect of berberine on anti-cancer potential of innate immune cells mainly macrophages, the macrophages were treated with crude soluble antigen (CSA) for 12 hrs followed by berberine for next 24 hrs. We have observed that CSA activated macrophages induces the release of pro-inflammatory cytokines IL-1β, TNFα and IL-12 which were enhanced in the presence of berberine from CSA activated macrophages and no significant change was observed in the immunosuppressive cytokine (TGFβ and IL-10) (Fig1A-F). The macrophages were pre-treated with CSA showed high NO release, which was enhanced in the presence of berberine (Fig1E).

Berberine enhances CD4$^+$ T cell proliferation in CSA primed macrophages:

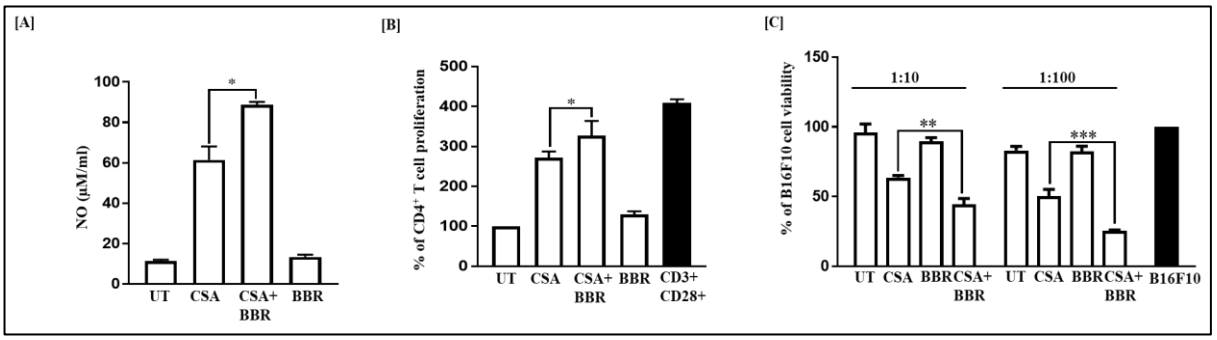


Fig.2: Raw264.7 (murine mouse macrophages) were pre-treated with crude soluble antigen (CSA) for 15 hrs followed by berberine (10µg/ml) treatment for 15 hrs. The supernatant was collected to analyse NO using Griess method [A]. The bone-marrow derived macrophages were pre-treated with CSA and berberine for 24 hrs followed by co-culture with splenic CD4+ T cells for 72 hrs and analysed for proliferation by MTT assay [B]. The bone-marrow derived macrophages were pre-treated with CSA and berberine for 24 hrs followed by co-culture with Total T cells for 72 hrs. Later the activated T cells were co-cultured with B16-F10 melanoma cells in the ratio of 1:10 and 1:100 and checked for viability using MTT assay [C]. Data

*are of three experiments and represented as mean ± SEM. Statistical significance was determined by One-way ANOVA with Turkey's multiple comparison (***p < 0.0001; **p< 0.001; *p < 0.05).*

To check the immune modulation potential of berberine on adaptive immune cells, we have co-cultured CSA-primed macrophages with CD4 positive T cells in the presence or absence of berberine. Here in we have observed that treatment with CSA potentiated CD4 T cell proliferation, which was enhance by berberine (Fig 2A). Later, total T cells co-cultured with macrophages, which was prior primed with CSA and berberine in the ratio of 1:10. The activated T cells co-cultured with B16F10 melanoma cells at different ratios. We have observed that berberine in the presence of CSA reduced the B16-F10 melanoma cell proliferation, thus enhancing the CTL activity of CD8 T cells (Fig 2B).

Berberine prevents immunosuppression but promotes inflammatory cytokines production:

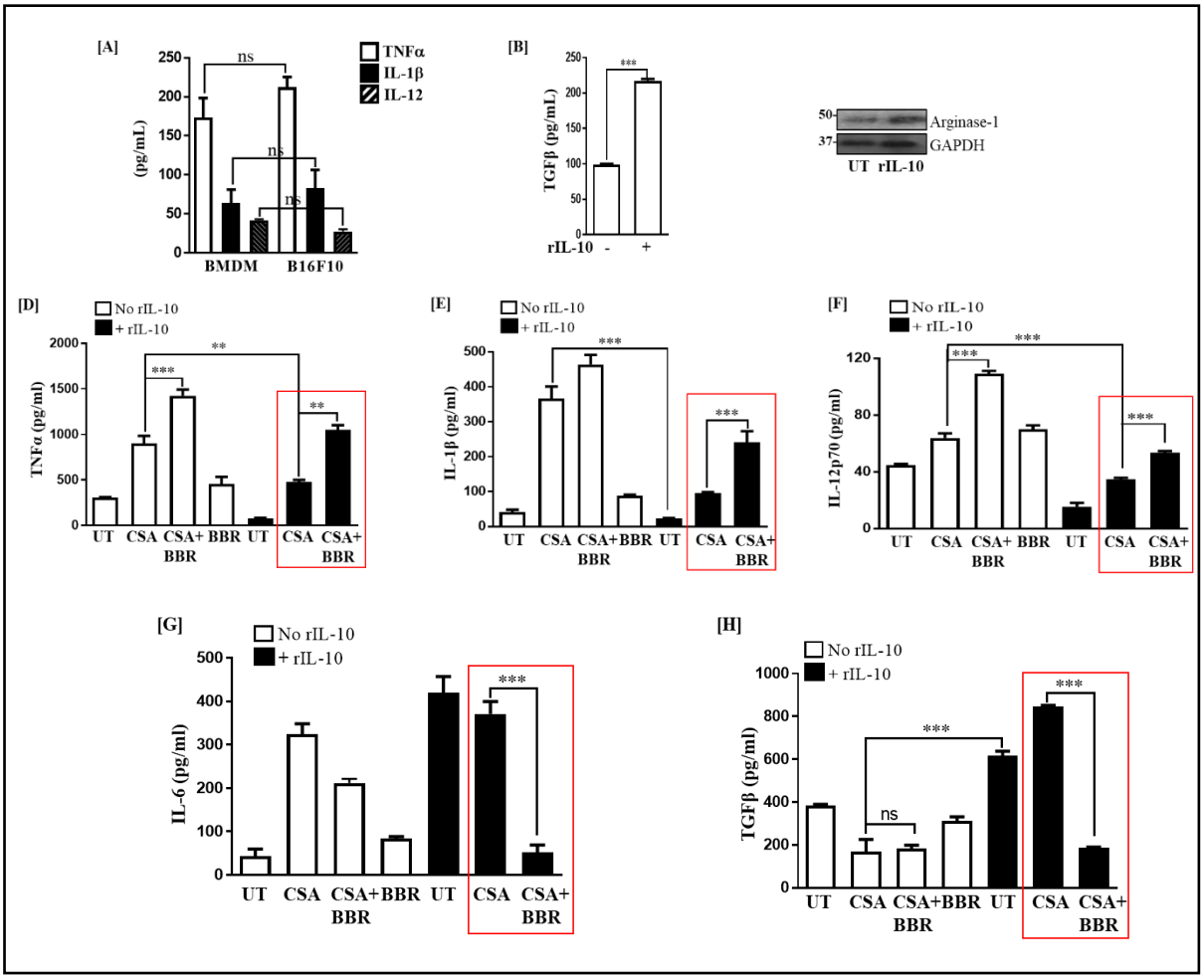
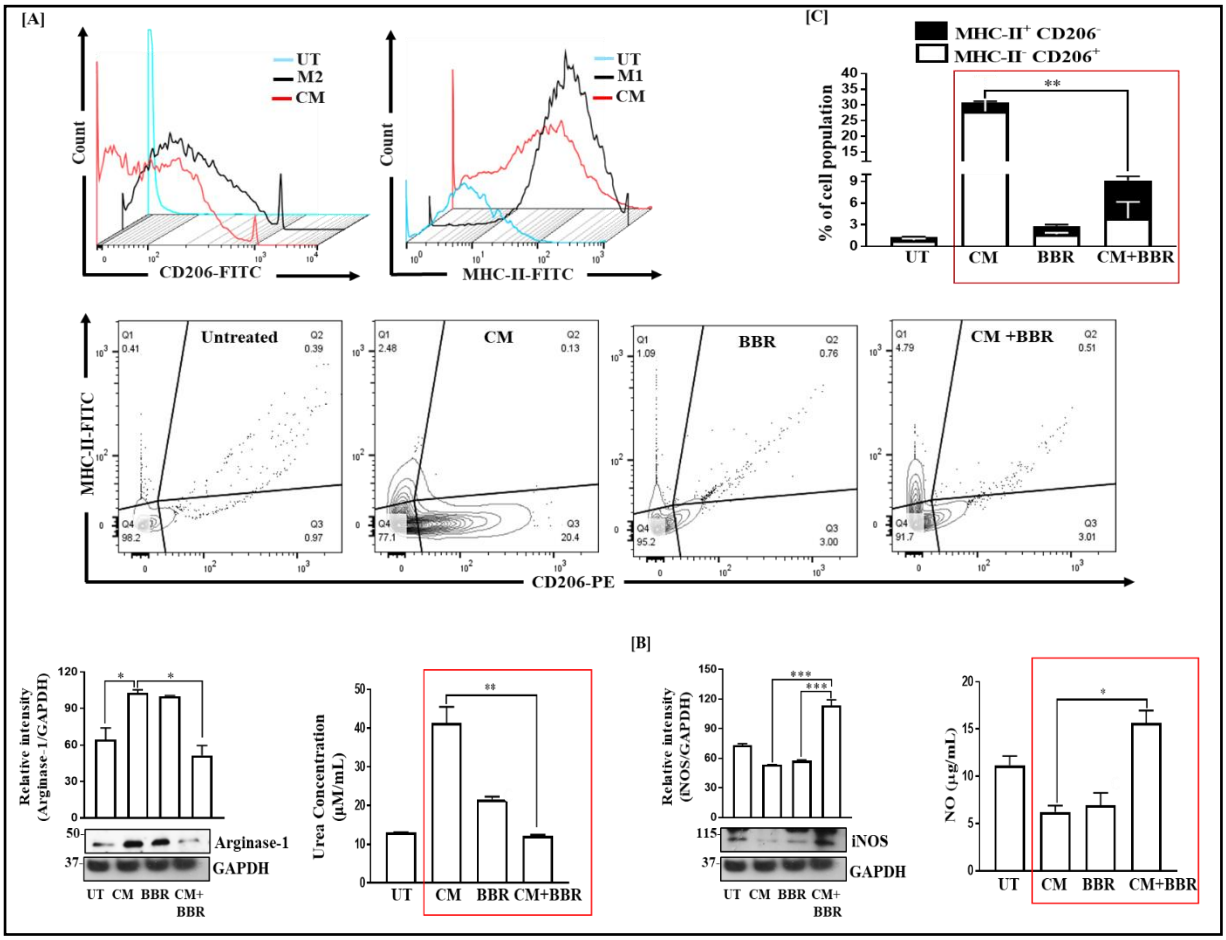


*Fig.3: The media from the BMDM (M0) and confluent B16F10 cells was harvested and analyzed for TNFα, IL-1β and IL-12 by ELISA [A]. To confirm the generation of the immunosuppressive environment in presence of rIL-10, BMDM were treated with rIL-10 (20ng/mL) for 12 hrs, cells were collected and checked for Arginase-1 expression by western blotting and supernatant was used to assess TGFβ release by ELISA [B and C]. BMDMs were pre-treated with rIL-10 for 12 hours followed by washing and treatment with CSA (10μg/mL) for 24 hours. Later the cells were treated with berberine (10μg/ml) for next 24 hours and various cytokines TNFα, IL-1β, IL-12p70 cytokine levels were analysed using ELISA [D-F]. BMDMs were pre-treated with rIL-10 (20ng/ml) for 12 hours followed by washing and treatment with CSA (10μM) for 24 hours. Later the cells were treated with berberine (10μg/ml) for next 24 hours and various cytokines IL-6, TGFβ cytokine levels were analysed using ELISA [G and H]. Data are of three experiments and represented as mean ± SEM. Statistical significance was determined by One-way ANOVA with Turkey's multiple comparison (***p < 0.0001; **p< 0.001; ns= non significant).*

The cancer cells secrete various factors that impart immunosuppressive properties to macrophages creating a suppressive intra-tumoral milieu (Sousa et al., 2015). Here, we examined whether berberine would prevent the generation of the immunosuppressive microenvironment. We have checked the levels

of pro-inflammatory cytokines from BMDM and B16F10 culture supernatant (Fig.3A). To understand the effect of IL-10 mediated immune-suppression, the macrophages were pre-treated with rIL-10 for 12 hrs, the generation of immunosuppressive microenvironment was confirmed by increased TGF-β but reduced pro-inflammatory cytokines (TNFα, IL-1β) and IL-12 release (Fig.3B-F). We observed that crude soluble antigen/tumor antigen (CSA) primed macrophages released pro-inflammatory cytokines (Fig.3D-F). To understand the effect of IL-10 mediated immune-suppression, the macrophages were pre-treated with rIL-10 for 12 hrs in presence/absence of CSA and berberine. In the presence of rIL-10, CSA-primed macrophages demonstrated increased release of immunosuppressive cytokines: IL-6 and TGF-β (Fig.3G and H) while reduced release of pro-inflammatory cytokines and IL-12 (Fig.3F). This indicates that macrophages are capable of mounting pro-inflammatory response until they encounter immunosuppressive conditions. Berberine significantly reduced rIL-10-induced IL-6 and TGF-β release from CSA-primed macrophages and rescued the release of IL-1β, TNFα and IL-12 (Fig.3D-H). Berberine thus prevented IL-10-induced suppression to restore inflammatory microenvironment.

Berberine reverts the generation of TMC-induced TAM -like M2 macrophages.



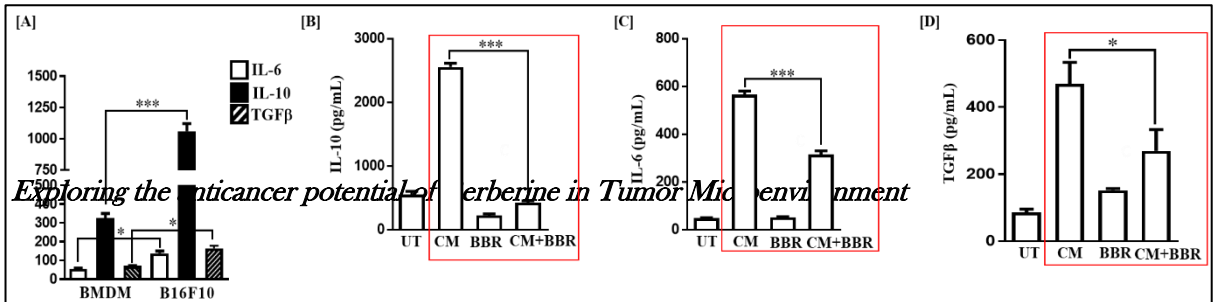


*Fig.4 BMDMs were cultured in presence of CM (culture supernatant of B16F10 + crude soluble antigen)/ IL-4 and IL-10 (M2)/ IFN-γ (M1) for 5 days were analysed for CD206 and MHC II expression by flow cytometry [A]. BMDM were incubated in CM for 5 days followed by treatment with berberine for 24 hr and analysed for CD206 and MHC-II expression by flow cytometry [B]. The percentage of MHC-II+CD206- and MHC-II-CD206+ cell population is plotted. The statistical significance of MHC-II- CD206+ is shown in [C]. BMDM were incubated in CM for 5 days followed by treatment with berberine for 24 hr and analysed for iNOS and Arginase-1 expression by western blotting. GAPDH was used as an internal control. BMDM were incubated in CM for 5 days followed by treatment with berberine for 24 hr and analysed for urea via Arginase-1 activity and NO release by Griess method [A and B] The media from the BMDM (M0) and confluent B16F10 cells was harvested and analyzed for IL-6, TGFβ and IL-10 by ELISA [A]. BMDM were incubated in CM for 5 days followed by treatment with berberine for 24 hr and analysed for various pro-inflammatory (IL-1β, IL-12, TNFα) and anti-inflammatory cytokines (IL-10, IL-6, TGFβ) by ELISA [B-G]. Data are of three experiments and represented as mean ± SEM. Statistical significance was determined by Two-way ANOVA with Turkey's multiple comparison (**p < 0.001)*

Macrophage plasticity is the distinctive feature of the TME leading to immune tolerance. Various cytokines secreted from tumor at TME play an important role in macrophage polarization. In order to depict the *in vivo* microenvironment consisting of cellular antigens and secretome, the tumor mimicking conditions (CM) consisting of CSA and conditioned media were provided to cells. To check if CM drives macrophages towards TAM-like M2 phenotype, BMDM were cultured in CM for 5 days. The M1/M2 macrophages generated using the established protocols showed high expression of MHC-II and CD206 in the M1 and M2 phenotype respectively. While BMDM in presence of CM showed high CD206 expression and low MHC-II expression demonstrating generation of TAM-like M2 phenotype (Fig.4A). Later, the generated macrophages were treated with berberine to test whether it can revert the TAM-like M2 phenotype into a functional M1 macrophages. The flow cytometric analysis showed that $CD206^+$ cell population was higher in CM induced macrophages indicating the generation of TAM-like M2 phenotype. Surprisingly, berberine reverted CM-induced TAM-like M2 as observed by decreased $CD206^+$cells and increased in $MHC\text{-}II^+$ cells. (Fig.4B and C). We observed an increase in Arginase-1 protein expression with increase in urea concentration in the CM treated macrophages also suggested the generation of TAM-like M2 phenotype, which was abrogated in presence of berberine. Simultaneously, an increase in the iNOS expression was observed in berberine treated TAM-like macrophages which was evident with NO release (Fig. 4D and E). It was further confirmed via decreased release of IL-10, IL-6 and TGF-β following berberine treatment (Fig.4F-H). Moreover, berberine treatment potentiated the release of TNFα and IL-1β in presence of CM (Fig.4I and J). The results demonstrate that berberine reverts TAM-like M2 phenotype to a functional M1-like macrophages.

Berberine rescues CM-induced immune suppression in M1 macrophages.

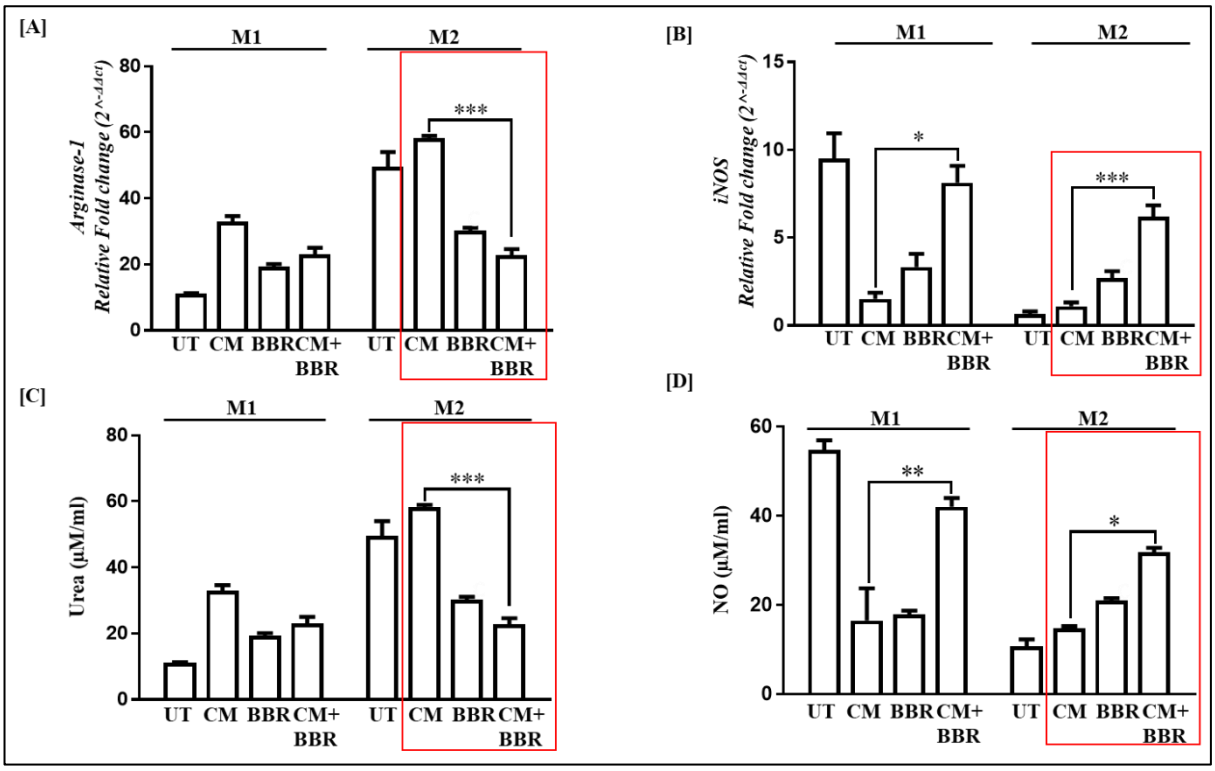


*Fig.5 . Polarized macrophages were incubated in CM for 5 days with/without berberine treatment for 6hrs and analysed for Arginase-1 and iNOS transcripts through qPCR analysis and plotted as 2-ΔΔCT values [A and B]; Polarized macrophages were incubated in CM for 5 days with/without berberine treatment for 24hrs analysed for urea by Arginase-1 activity [C] and NO assay by Griess reagent [D]. Data are of three experiments and represented as mean ± SEM. Statistical significance was determined by Two-way ANOVA with Turkey's multiple comparison (***p < 0.0001; **p < 0.001; *p < 0.05)*

During initial stages of tumor development, tumor site is ubiquitous system with mixture of M1 and M2 macrophages. But as tumor progresses, the secretory factors influence macrophages to most likely altered to a TAM-like M2 phenotype. Hence, we wanted to understand the effect of berberine on polarized macrophages maintained in CM. We observed that CM promoted TAM-like M2 phenotype in M1 and M2 macrophages, which was evident by increased arginase-1 transcripts and urea release. However, berberine reverted the phenotype of M2 macrophages to low arginase-1 and high iNOS expressing subset indicating the reversal to M1 macrophages (Fig.5A-D).

Berberine enhances the release of pro-inflammatory cytokines from CM-induced M2 macrophages

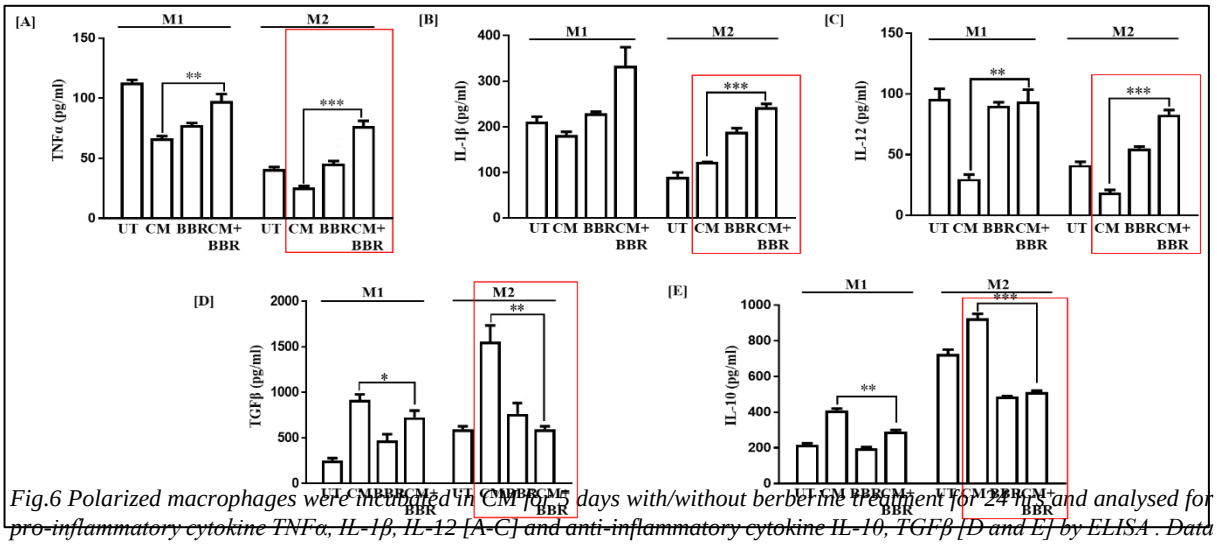


Fig.6 Polarized macrophages were incubated in CM for 5 days with/without berberine treatment for 24 hrs and analysed for pro-inflammatory cytokine TNFα, IL-1β, IL-12 [A-C] and anti-inflammatory cytokine IL-10, TGFβ [D and E] by ELISA . Data

*are of three experiments and represented as mean ± SEM. Statistical significance was determined by Two-way ANOVA with Turkey's multiple comparison (***p < 0.0001; **p < 0.001; *p < 0.05)*

The polarized macrophages were cultured in CM for 24 hrs in the presence/absence of berberine. The supernatant was used to check for various cytokines release. M1 macrophages showed high release of pro-inflammatory cytokines (TNFα, IL-1β, IL-12) while when cultured in CM, there was an increase in the immunosuppressive cytokines indicating that macrophages were skewed to immunosuppressive phenotype (TGFβ, IL-10). The maintenance of macrophages in the CM induced a significant amount of TGF-β and IL-10 release from the M2 macrophages and even from the M1 macrophages. Berberine reduced TGF-β and IL-10 release from both the subsets indicating a reversal from an immunosuppressive to an activated phenotype. In CM, both the subsets failed to induce the release of TNFα, IL-1β and IL-12 while berberine treatment facilitated TNFα, IL-1β and IL-12 release from M1 and even from M2 subset (Fig.6A-E). Considering the above results, berberine demonstrated restoration of inflammatory activity in macrophages even in CM-induced micro-environment, thus indicating that berberine could act as an effective drug during initial and later stages of tumor development.

Berberine enhances MHC-II expression in CM-induced macrophages

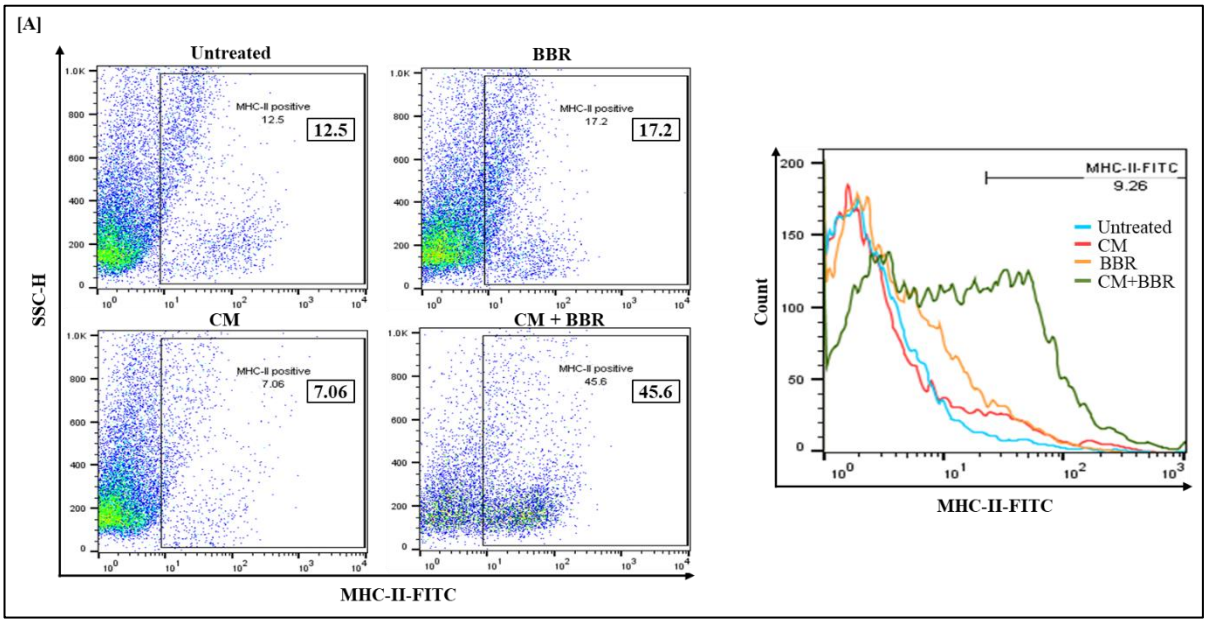


Fig.7 The BMDM were cultured in CM for 5 days. Later, the cells were treated with berberine for 24 hrs. The cells were collected and stained for MHC-II-FITC and analysed using Flow cytometry. The cells were gated using FSC vs SSC and gated population was analysed for MHC-II positive cells [A].

Antigen presentation is one of the important characteristics of the macrophages. For T cell activation, three signals are required: MHC-II-TCR; CD40-CD40L and CD80/86-CD28. TAMs display a defective MHC-II expression and impaired antigen presentation ability resulting in ineffective adaptive immune response. We wanted to check if berberine could enhance MHC-II expression in CM-induced macrophages. We have cultured macrophages in CM in the presence/absence of berberine and we have observed berberine enhanced MHC-II expression in CM-induced macrophages (Fig. 7A).

Berberine enhances CD40 expression in CM-induced macrophages

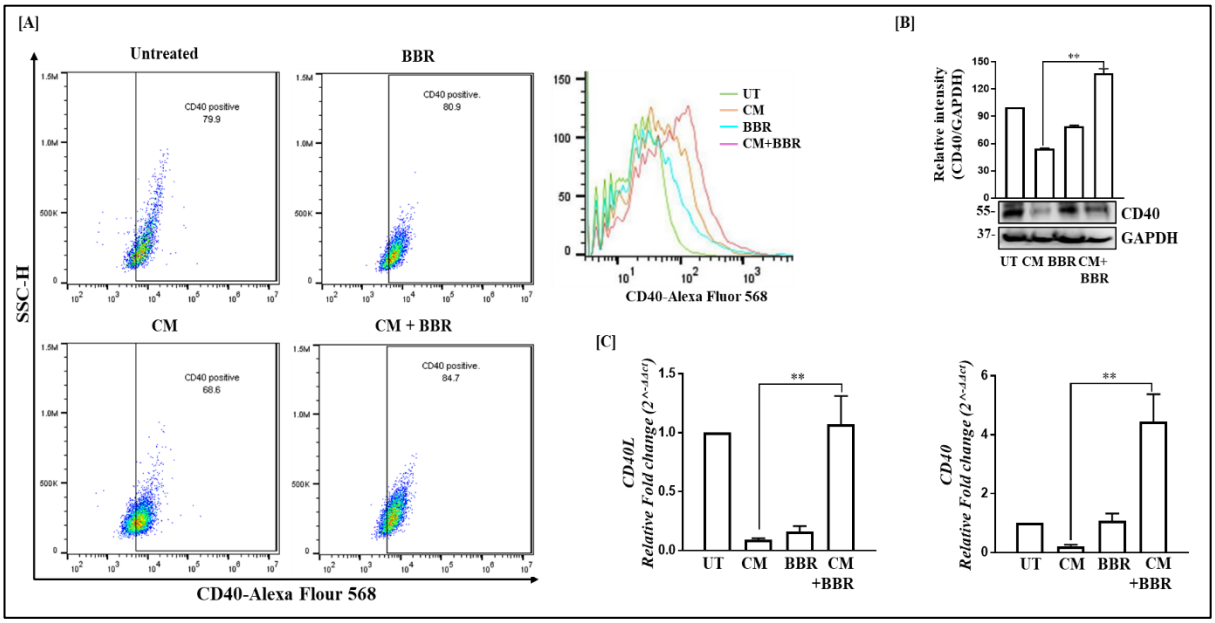


*Fig.8 The macrophages were pre-treated with conditioned media for 24 hrs followed by berberine treatment of 24 hrs. The cells were collected and stained for CD40-Alexa Flour 568 and analysed using Flow cytometry [A] and CD40 protein expression was analysed by western blotting [B]. The macrophages were pre-treated with conditioned media for 24 hrs. followed by berberine for 24 hrs. The cells were co-cultured with T cells for 12 hrs and checked for CD40 and CD40L by qPCR [C]. Data are of three experiments and represented as mean ± SEM. Statistical significance was determined by One-way ANOVA with Turkey's multiple comparison (**p < 0.001).*

CD40 is a co-stimulatory molecule that is present on antigen presenting cells and necessary for T cell activation. The macrophages were treated with CM and berberine for 24 hrs and analysed for CD40 expression. We have observed that berberine enhances CD40 expression in CM-induced immunosuppressive macrophages which was evident with increased CD40 protein and mRNA expression (Fig.8A-C). We have even observed an increase in CD40L transcripts on T cells. This data indicates berberine not only enhances MHC-II expression but also enhances CD40 expression in macrophages.

Berberine decreases PDL1 expression in CM-induced macrophages

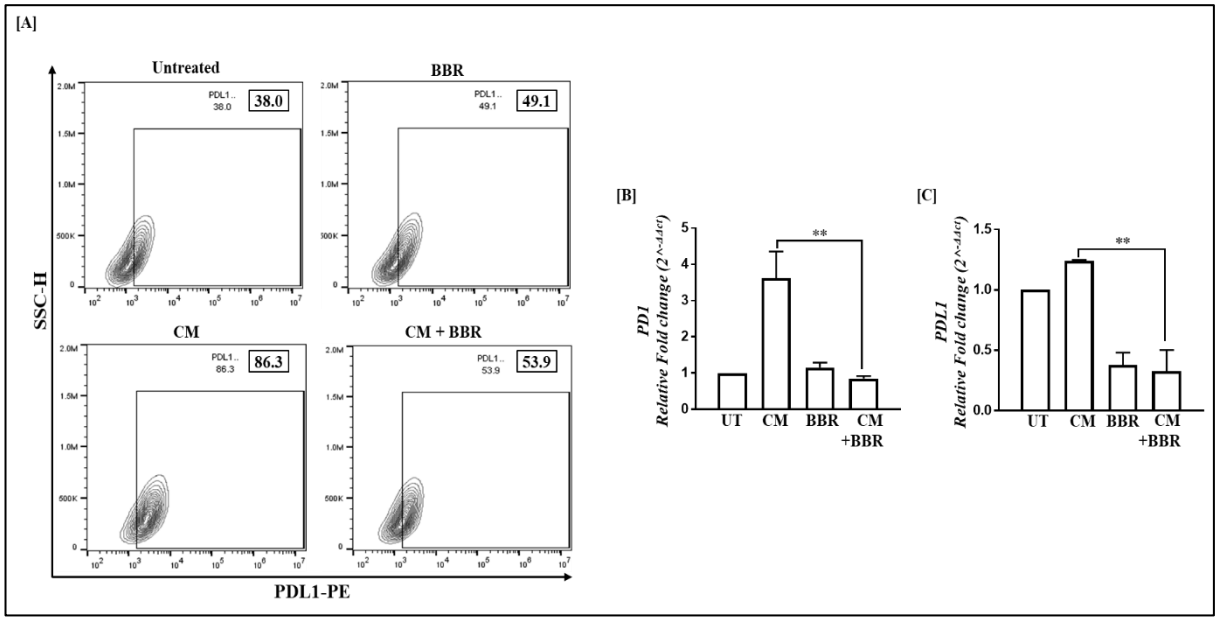


*Fig.9 The macrophages were pre-treated with conditioned media for 24 hrs followed by berberine treatment for 24 hrs. The cells were collected and stained for PDL1-PE and CD11b-APC analysed using Flow cytometry. The CD11b positive cells were further analysed for PDL1 expression and plotted [A]. BMDMs were pre-treated with conditioned media for 24 hrs followed by berberine treatment for 24 hrs. Later, the cells were co-cultured with CD4+ T cells for 12 hrs. The collected cells were analysed for co-stimulatory and co-inhibitory molecules by qPCR and plotted as 2-ΔΔCT values [B-E]. Data are of three experiments and represented as mean ± SEM. Statistical significance was determined by One-way ANOVA with Turkey's multiple comparison (**p < 0.001).*

PDL1 is a co-inhibitory molecule that mainly plays an important role in downregulating T cell immune response leading to T cell anergy or exhaustion. In tumor microenvironment, tumor associated macrophages upregulates PDL1 to downregulate T cell immune response in order to aid tumor progression. We have observed that CM-induced macrophages had high expression of PDL1 compared to untreated indicating that CM-induced macrophages are of immunosuppressive phenotype, while in the presence of berberine, the expression of PDL1 decreased (Fig. 9A-C). Thus, we have observed that berberine decreases the expression of co-inhibitory molecule while enhancing the co-stimulatory molecule CD40.

Berberine enhances T cell proliferation in CM-induced immune suppressive milieu.

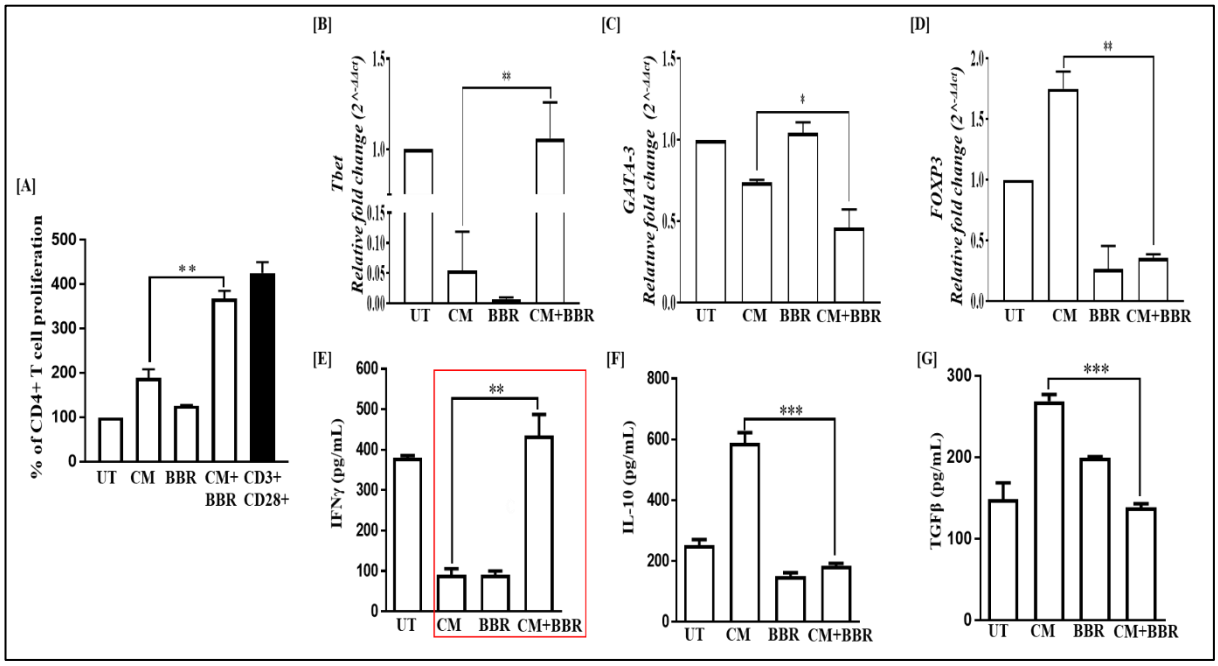


*Fig.10 BMDM were pre-treated with CM and berberine for 24 hrs and co-cultured with CD4+ T cells isolated from spleen of naïve mice for 72 hrs and analysed for T cell proliferation by MTT assay [A], and the collected supernatant was analysed for IFNγ, IL-10 and TGFβ by ELISA [E-G]. BMDM were pre-treated with CM and berberine for 24 hrs and co-cultured with CD4+ T cells isolated from spleen of the naïve mice for 12 hrs. The collected cells were analysed for T cell transcription factors by qPCR and plotted as 2-ΔΔCT values [B-D]. Data are of three experiments and represented as mean ± SEM. Statistical significance was determined by One-way ANOVA with Turkey's multiple comparison (***p < 0.0001; **p < 0.001; *p < 0.05)*

We wanted to check if treatment with berberine enhanced adaptive immune response in TME. We have co-cultured CM-induced macrophages with T cells in the presence/absence of berberine for 72 hrs. We have observed proliferation in CD4+ T cells co-cultured with CM-induced macrophages, which was enhanced in the presence of berberine. In addition to that, we have observed an increase in GATA-3 and FOXP3 transcripts from CM-induced macrophages which was evident with increased IL-10 and TGFβ. The treatment with berberine decreased GATA-3 and FOXP3 transcripts along with increase in Tbet and IFNγ release. This data indicates that T cell proliferation in CM treatment enhanced Th2 or regulatory T cells which was abrogated in the presence of berberine.

Berberine decreases IL-10 and IL-4 release from CD4$^+$ T cells co-culture with M2 polarised macrophages

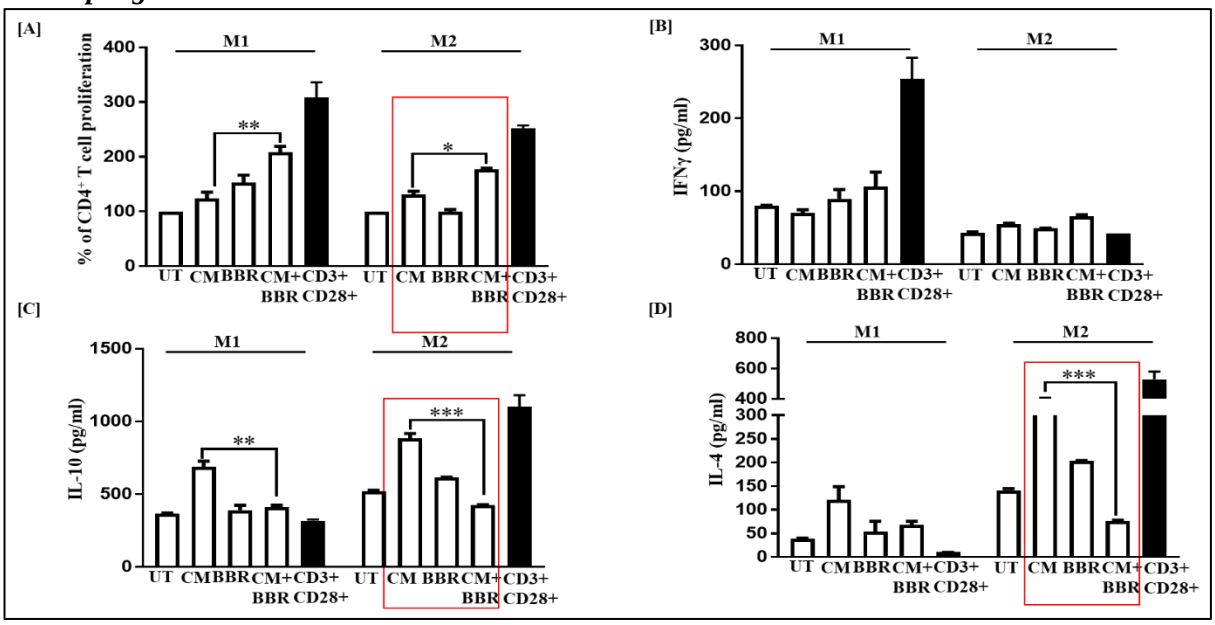


*Fig.11 Polarized macrophages were pre-treated with CM and berberine for 24 hrs and co-cultured with CD4+ T cells isolated from spleen of the naïve mice for 72 hrs and analysed for T cell proliferation by MTT assay [A], and the collected supernatant was analysed for IFNγ, IL-10 and IL-4 by ELISA [B-D]. Data are of three experiments and represented as mean ± SEM. Statistical significance was determined by Two-way ANOVA with Turkey's multiple comparison (***p < 0.0001; **p < 0.001; *p < 0.05)*

Since the TME consists of both M1 and M2 macrophages, we polarised the macrophages to M1 and M2 phenotype and co-cultured with CD4$^+$ T cells. We have observed an increased release of IL-10 and IL-4 from M1 and M2 polarised macrophages in CM. The treatment with berberine decreases IL-10 and IL-4 release from CM-induced polarised macrophages. This indicates that treatment with berberine may skew CD4 T cells to low IL-10 and IL-4 secreting cells when co-cultured with CM-induced macrophages.

Berberine enhances CTL activity and decreases cellular viability of B16-F10 melanoma cells

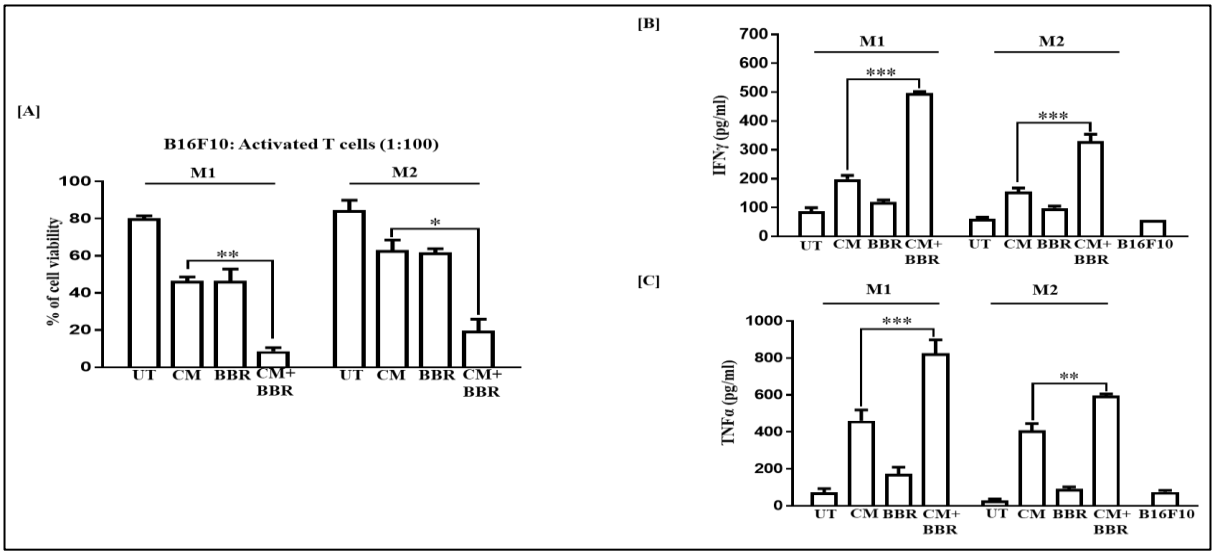
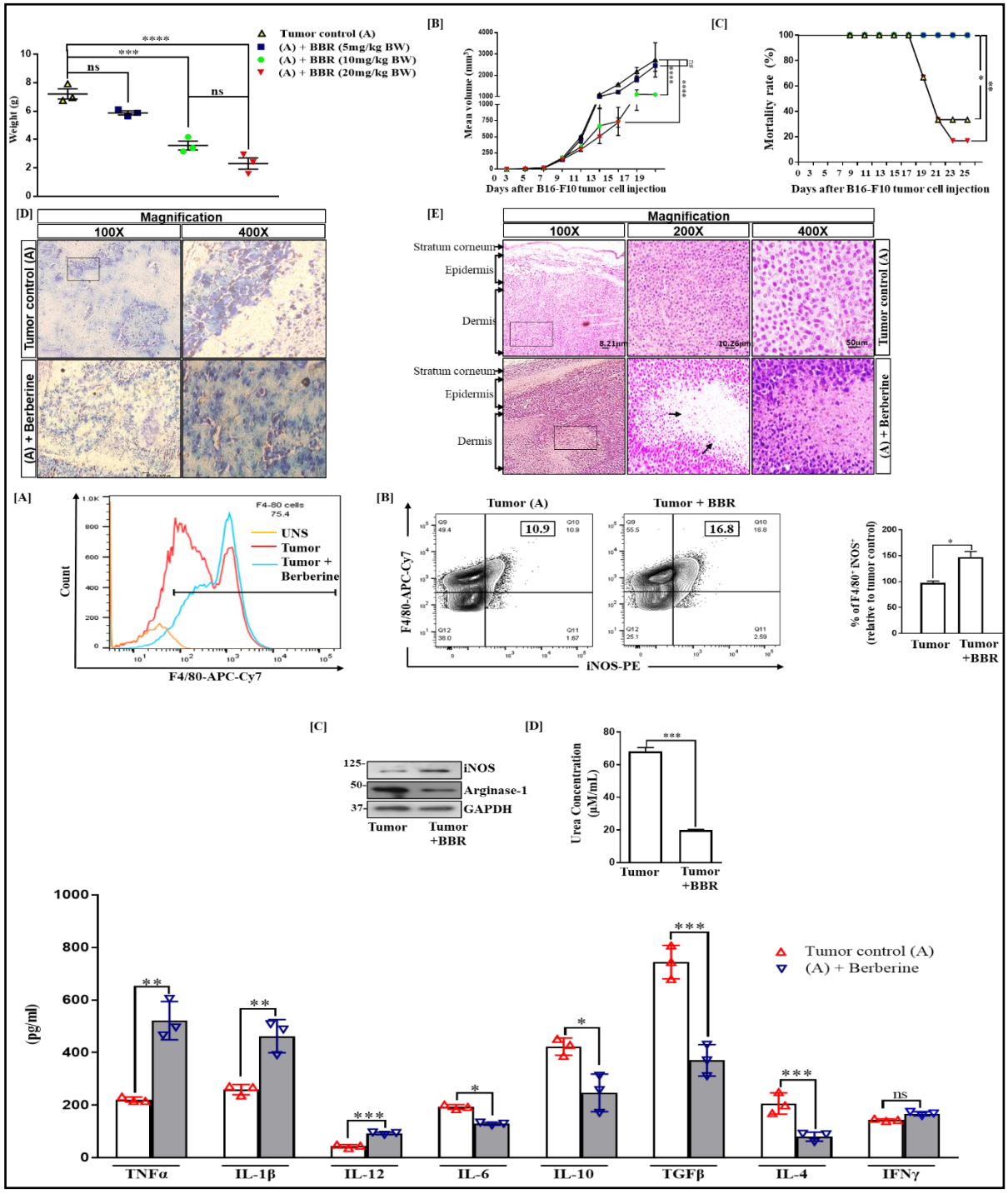


Fig. 12 Polarized macrophages were pre-treated with CM and berberine for 24 hrs and co-cultured with Total T cells isolated from spleen of the naïve mice for 72 hrs. Later, the collected cells were co-cultured with B16F10 cells in different ratios and

*the cellular viability was analysed by MTT assay [A]. The supernatant was collected and scored for IFNγ [B] and TNFα [C] by ELISA. Data are of three experiments and represented as mean ± SEM. Statistical significance was determined by Two-way ANOVA with Turkey's multiple comparison (***p < 0.0001; **p < 0.001; *p < 0.05)*

CD8 T cells play an important role in tumor surveillance. We wanted to check if treatment with berberine could enhanced CTL activity. The polarised macrophages were co-cultured with total T cells and analysed for cellular viability by MTT. We have observed that CM-induced polarised macrophages showed decreased cell viability of B16-F10 cells in M1 as well as M2 polarized macrophages, which was further decreased in the presence of berberine. Furthermore, treatment with berberine in CM induced polarized macrophages enhanced IFNγ and TNFα, thus increasing the cytotoxic activity of T cells. This indicates that berberine aids in cytolytic activity of T cells.

Berberine decreases tumor growth in a dose dependent manner



Exploring the anticancer potential of Berberine in Tumor Microenvironment

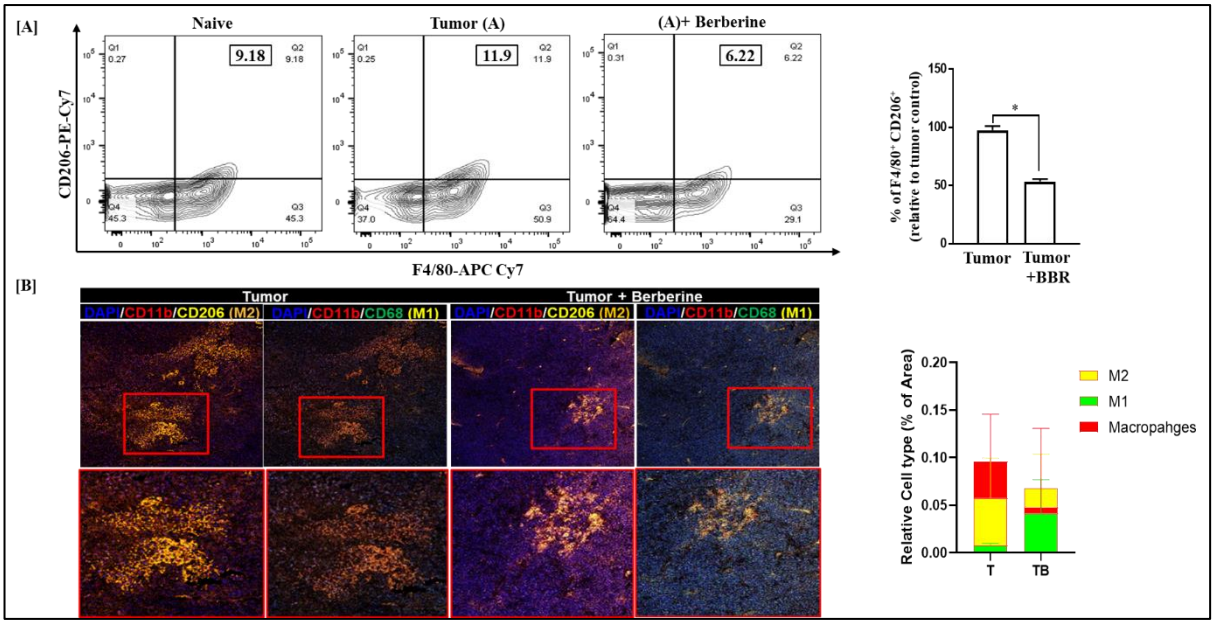


*Fig. 13 The weight and volume of the tumor tissue from the control and berberine treatment group was measured and plotted in [A and B]. The percentage of survival rate of the tumor bearing mice is plotted as [C]. The tumor tissue was stained for Giemsa and H&E staining and images were shown in [C] The tumor tissue were excised from the control and treatment group of tumor bearing mice and analysed for F4/80-APC Cy7 by flow cytometry and plotted as histogram [A]. The excised tumor tissue from the control and treatment group of tumor bearing mice was analysed for F4/80-APC Cy7 and iNOS-PE (M1) by Flow cytometry [B]. The tumor tissues were minced and the extracted protein was used to analysed for iNOS and Arginase-1 proteins using western blotting [C] and urea release by Arginase-1 activity [D]. The spleens from the tumor bearing mice were collected and analysed for F4/80-APC Cy7 and CD206-PE Cy7 (M2) by flow cytometry and plotted as % of F4/80+ CD206+ cell population [A]. The sections of 4 microns was prepared from the excised tumor tissue and stained for CD206-FITC, CD11b-APC, CD68-PE and DAPI and observed under Leica confocoal microscopy. The excised tumor tissue from control and berberine treatmet group was homogenized in the media and the supernatant was used to score cytokines (TNFα, IL-1β, IL-12, IL-6, IL-10, TGFβ, IL-4, IFNγ) by ELISA. Data are of three experiments and represented as mean ± SEM. Statistical significance was determined by Two-way ANOVA with Turkey's multiple comparison (***p < 0.0001; **p < 0.001; *p < 0.05; ns= non significant).*

To check the efficacy of berberine in regulating the macrophage polarization *in vivo*, the syngeneic model for melanoma was established. The dosage of berberine was optimized in C57BL/6 tumor bearing mice. Berberine did not cause any significant visible physiological changes in tumor bearing mice and non tumor bearing mice other than tumor reduction. Non tumor bearing C57BL/6 mice did not show toxicity till 40mg/kg of body weight. The weight and volume of tumor in berberine treatment group was significantly reduced compared to the tumor control group. It improved the survival of tumor bearing mice. H & E staining of the tumor tissues in the control and berberine treatment group shows tissue destruction in the berberine treatment group as evident by deteriorated stratum corneum, epidermis and dermis layers. In addition to that, Giemsa staining indicated increased immune cell recruitment to the tumor site in the berberine treatment group. Cells collected from the excised tumor of both groups were stained for F4/80 and iNOS to determine the classically activated macrophage population. We observed an increase in F4/80+ iNOS+ cells in berberine treated group compared to tumor group. This was also supported by the increased distribution of CD68 expression on CD11b macrophages in berberine treated tumor tissue compared to tumor control. We further analysed cytokine profiling from tumor and serum of the tumor bearing control mice and berberine treated tumor mice. We observed high levels of IL-6, IL-10 and TGFβ in the tumor tissue, which was reduced in the berberine treated group. Similarly, increased IL-6 and IL-10 was observed in sera of the tumor bearing mice which was decreased by berberine treatment. To further understand the underlying mechanism of macrophage polarization, we analysed the protein expression of Arginase-1 and iNOS from tumor samples of the control and treatment group. The berberine treated group showed decrease in Arginase-

1 expression while it showed increased expression of iNOS. Moreover, there was significant decrease in the Arginase-1 activity from the tumor tissue of the berberine treatment group compared to control group. This suggest that berberine not only inhibited the tumor growth and improved survival in tumor bearing mice but also indicated the effect can partly be mediated by function restoration to high iNOS expressing M1macrophages and subsequent immuno-activation.

Berberine attenuates the frequency of MDSC in tumor tissue

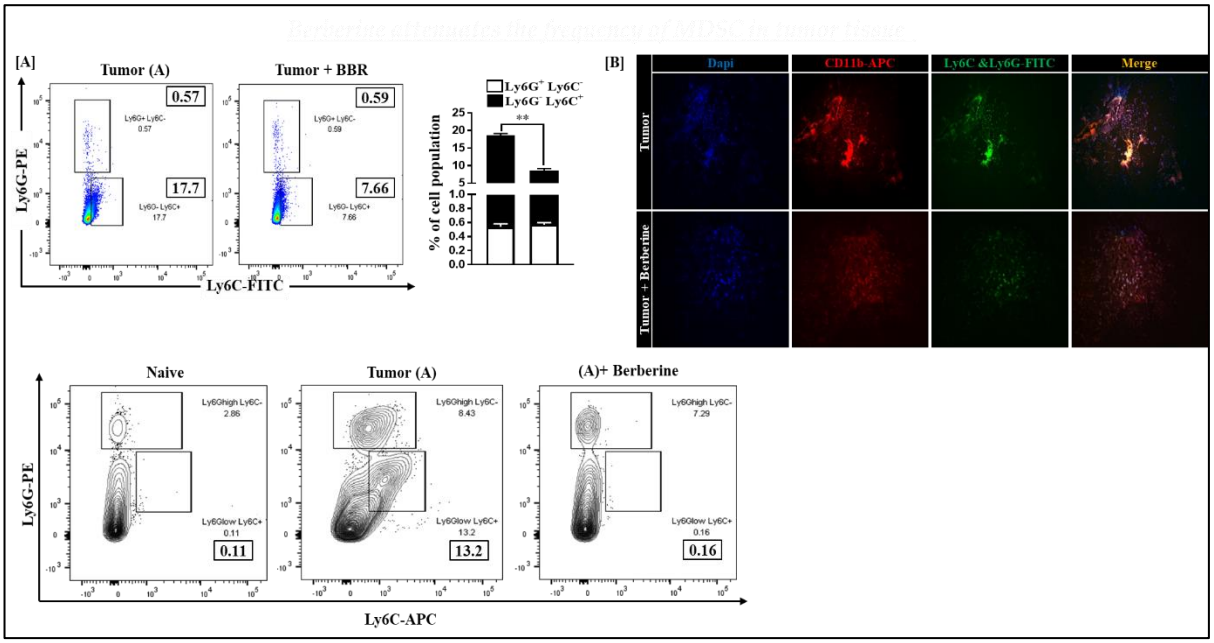


*Fig.14 The excised tumor tissue from control and berberine treatment group were stained for Ly6G-PE andLy6C-FITC for MDSC and analysed by flow cytometry and plotted as percentage of Ly6G + Ly6C- and Ly6G- Ly6C + cells [A]. Sections of 4microns was prepared from tumor tissues and stained for CD11b-APC, Ly6C-Ly6G-FITC and DAPI, and observed under Fluorescence microscopy [B]. The splenic tissues from control and treatment groups of tumor bearing mice were analysed for MDSC population by flow cytometry. The CD11b positive cells were further stained to Ly6C-APC and Ly6G-PE and analysed using flow cytometry [C]. Data are of three experiments and represented as mean ± SEM. Statistical significance was determined by Two-way ANOVA with Turkey's multiple comparison (**p < 0.001).*

Myeloid Derived Suppressor Cells (MDSC) are an immunosuppressive subset of myeloid cells that polarised to macrophages in accordance to surrounding milieu. We have observed that treatment with berberine reduces the expression of Ly6C and Ly6G indicating the decrease in MDSC generation. Similarly, the splenic macrophages even showed decrease in Ly6C and Ly6G, which are markers for granulocytic and monocytic MDSC. This indicates that treatment with berberine decreases the recruitment of MDSC in tumor bearing mice.

Berberine reduces regulatory T cell population in vivo

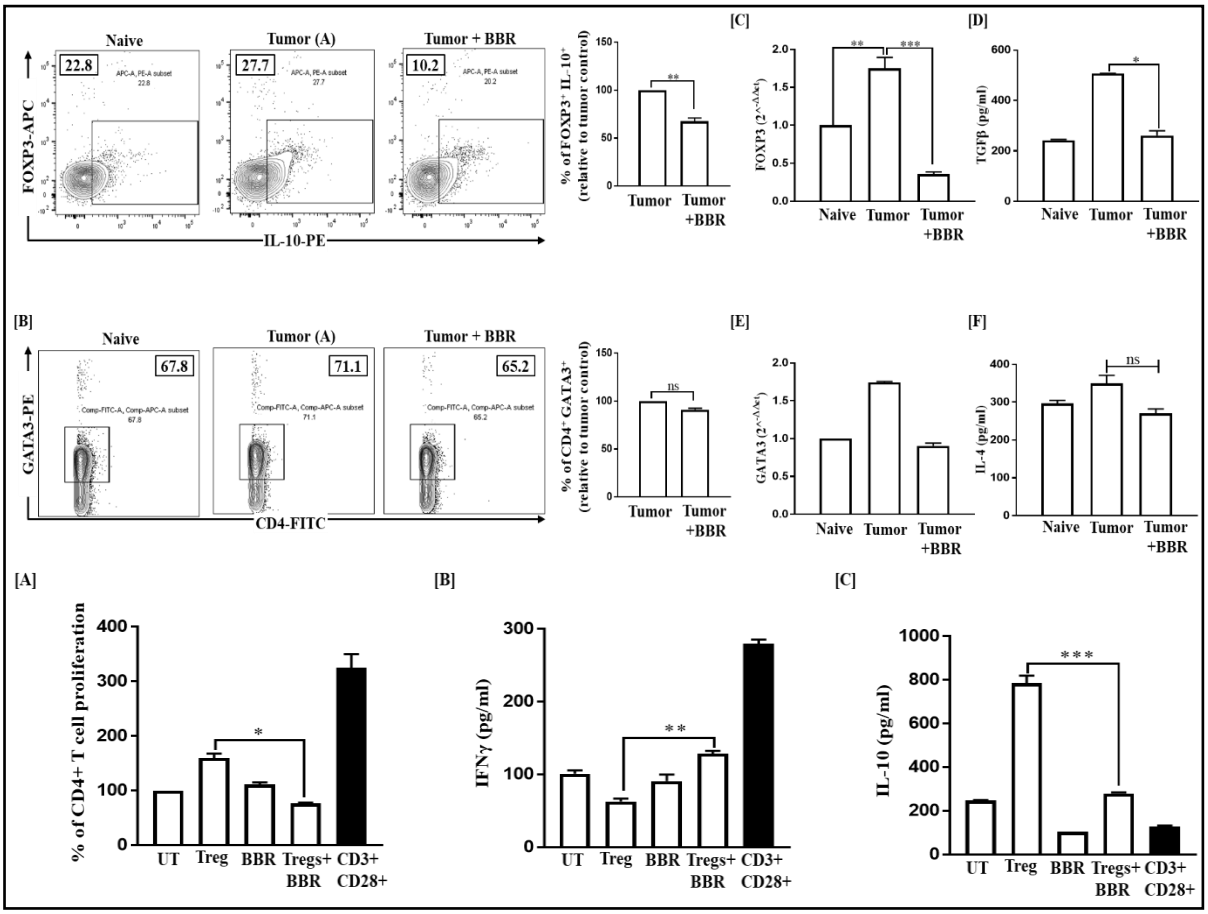


*Fig.15 The tumor tissues from mice of both groups were minced and the pooled cells were stained for FOXP3-APC and IL-10-PE and analysed by flow cytometry and plotted as percentage of FOXP3 + IL-10+ cells [A] and GATA3-PE and CD4-FITC and analysed by flow cytometry and plotted as percentage of GATA3 + CD4+ cells [B]. RNA was isolated from the excised tumor of control and berberine treated group and analysed for key T cell transcription factors by qPCR normalized to β actin and plotted as 2-ΔΔCT values [C and E} The tumor tissue was homogenized in the media and the supernatant was used to score cytokines by ELISA [D and F]. The BMDM were pre-treated with berberine, anti-CD3 and anti-CD28 for 24 hrs. and then co-cultured with regulatory T cells (generated using a known cocktail of TGFβ and retinoic acid) for 72 hrs. Later, the T cell proliferation was analysed by MTT assay [A]. The supernatant was collected and IFNγ and IL-10 were scored by ELISA [B and C]. Data are of three experiments and represented as mean ± SEM. Statistical significance was determined by Two-way ANOVA with Turkey's multiple comparison (***p < 0.0001; **p < 0.001; *p < 0.05).*

To check the effect of berberine on adaptive immune response *in vivo* in TME, we have observed an increase in the regulatory T cells in the presence of splenic tissue of tumor bearing mice as evident by increased FOXP3, IL-10 and TGFβ levels. Along with it, there was a slight increase in the GATA-3 (Th-2 subset) as well. This indicates that tumor microenvironment consists of high frequency of immunosuppressive CD4 T cells thus, skewing the microenvironment in its favour. But in the berberine treatment group, the frequency of regulatory T cells was decreased which was evident by decrease in TGFβ and IL-10. To further confirm the results, we have generated regulatory T cells in in vitro conditions e in the presence/absence of berberine. The generation of regulatory T cells was confirmed with low IFNγ and high IL-10 release. We have observed a decrease in the regulatory T cell proliferation in the presence of berberine. It was further confirmed by decrease in IL-10 and increased IFNγ. This indicates that berberine reduces functional aspect of regulatory T cells.

Berberine enhances T cell proliferation upon priming with tumor antigen

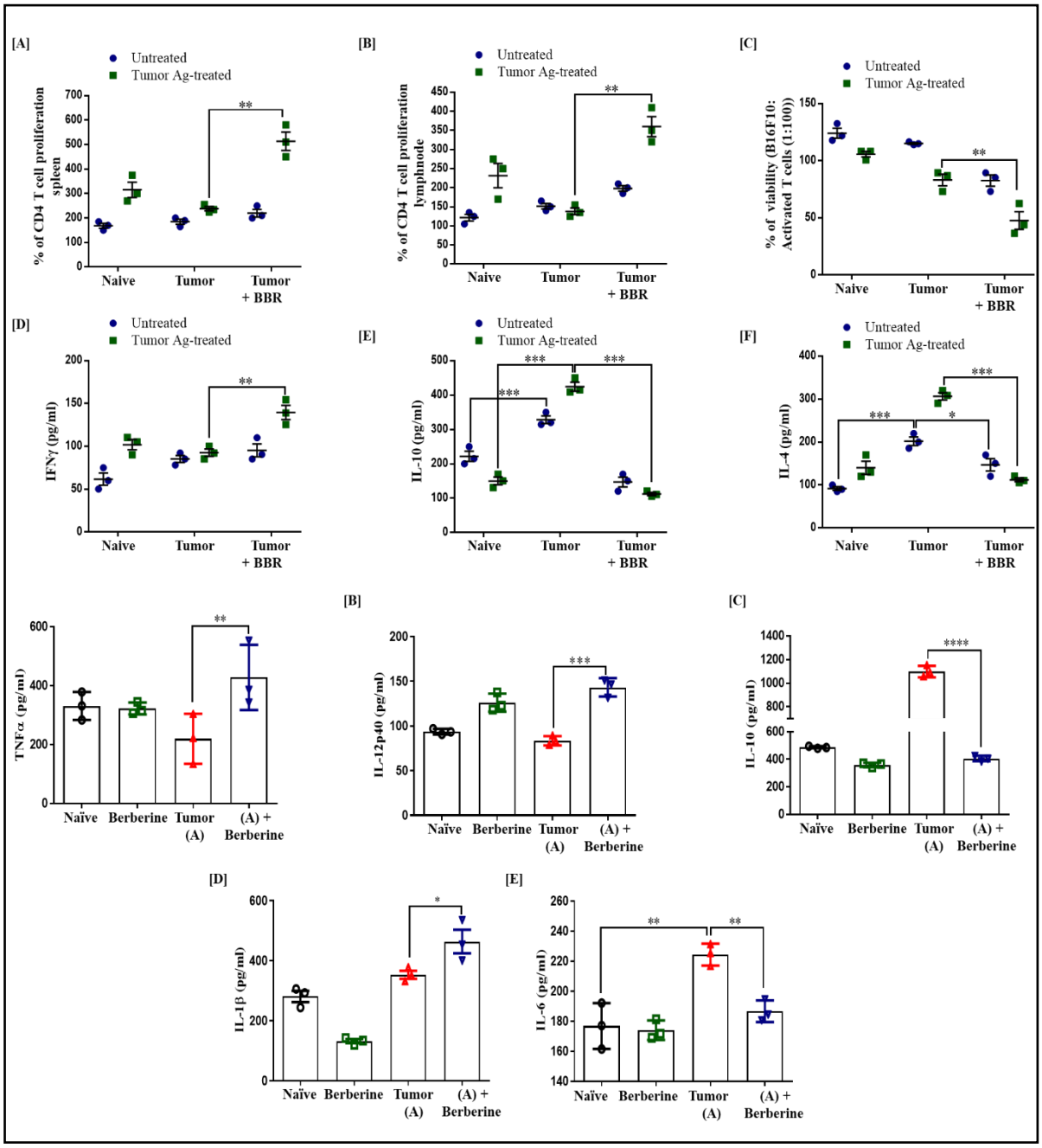


*Fig.16 The BMDM were primed with crude soluble antigen for 24 hrs and later co-cultured with the CD4+ T cells, isolated from the spleen and lymph nodes of tumor control and berberine treatment groups. The cellular proliferation of T cells was checked by MTT assay [A and B] and supernatant was used to score various cytokines by ELISA [D-F]. The BMDM were primed with crude soluble antigen for 24 hrs and later co-cultured with the Total T cells, isolated from the lymph nodes of tumor control and berberine treatment group for 72 hrs. Later these T cells were co cultured with B16F10 melanoma cells in 1:100 ratio for 5 days and cellular viability was checked using MTT assay [C]. The sera was isolated from blood collected from the control and treatment groups of tumor bearing mice and was used to check various cytokines by ELISA [A-E]. Data are of three experiments and represented as mean ± SEM. Statistical significance was determined by Two-way ANOVA with Turkey's multiple comparison (***p < 0.0001; **p < 0.001; *p < 0.05).*

In order to understand the effect of berberine in priming adaptive immune response. We have isolated T cells from lymph node and spleen of the tumor and treatment bearing mice and checked various cytokines. The macrophages were re-challenged with the tumor antigen, and co-cultured with T cells isolated from the lymph node and spleen of the tumor and treatment bearing mice. We have observed that T cells proliferate when co-cultured with T cells from tumor bearing mice which was enhanced in

the presence of berberine. We have observed that T cells from the lymph node of tumor bearing mice showed increased IL-10 and IL-4 while the berberine treatment group showed enhanced IFNγ. This indicates that T cell proliferation in the tumor bearing mice aids in immunosuppressive/tumor favouring subset which was abrogated in the berberine treatment group. It was further confirmed with elevated IL-12 and TNFα levels.

Berberine inhibits STAT-3 expression in cancer cells

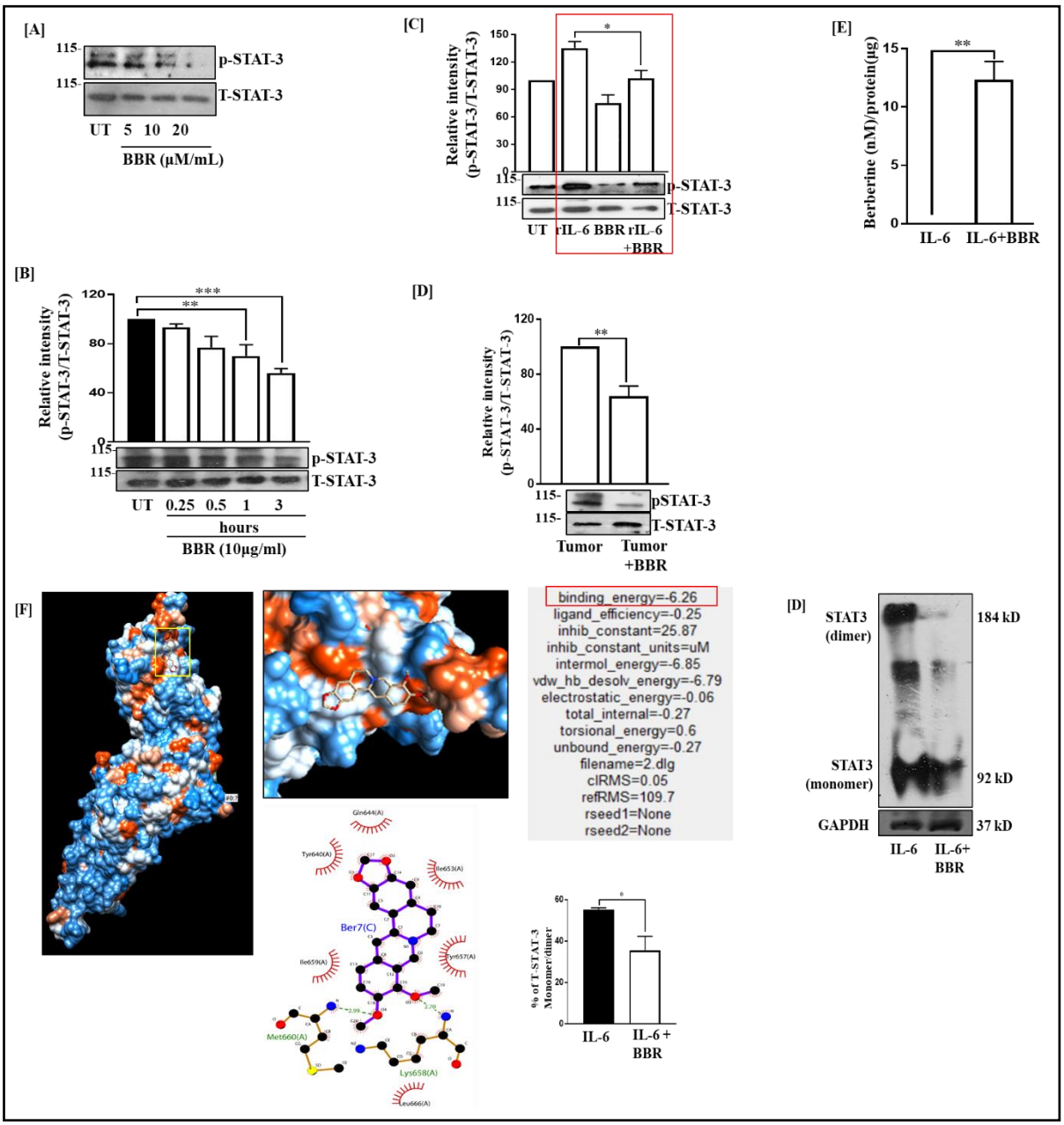


Fig.17 B16-F10 cells were treated w/o berberine for indicated time and dose and pSTAT-3 levels were assessed by Western blotting [A and B]. B16-F10 cells were supplemented with rIL-6 in presence /absence of berberine for 1hr and pSTAT-3 and T-STAT3 levels were analysed by Western blotting [C]. The tumor tissue from the control and berberine treatment groups were homogenised and the extracted protein was used to analyse STAT-3 expression by western blotting [D]. Cells were treated with berberine for 15min in w/o rIL-6. The cells were lysed and STAT3 were pooled by co-immunoprecipitation and analysed for STAT3-bound berberine at 240nm [E]. The interaction of STAT-3 (transcription factor) and berberine (ligand) is predicted using Autodock 4. The STAT-3 structure is based on the PDB entry 3CWG and berberine 3NP6. The interaction of berberine with STAT-3 protein at the SH2 domain is shown [F]. The Ligplot software indicates the presence of hydrogen bonds and hydrophobic interactions. Cells were treated with berberine for 30min in presence/absence of IL-6. The cells were lysed in cytoplasmic lysis buffer and protein was separated on blue-native gel and analysed for STAT3 monomers and dimers

*using western blotting [G]Data are of three experiments and represented as mean ± SEM. Statistical significance was determined by One-way ANOVA with Turkey's multiple comparison (***p < 0.0001; **p < 0.001; *p < 0.05).*

Multiple signals converge at the TME resulting in persistent activation of STAT-3. IL-6 and IL-10 are the most prominent released factors during cancer progression. IL-6 mediated STAT-3 phosphorylation induces IL-10 secretion which activates STAT-3 through autocrine signalling. Thus, signals form a triad of IL-6-STAT3-IL-10 axis that maintains a strong immunosuppressive microenvironment [22, 23]. We demonstrate that B16-F10 cells have high innate STAT-3 phosphorylation which was significantly inhibited following berberine treatment in a time- and dose-dependent manner. Additionally, berberine also inhibited rIL-6 induced STAT3 phosphorylation in B16-F10 cells. U.V spectrophotometric analysis of STAT3-bound berberine revealed that berberine binds to STAT3 protein, thus inhibiting its dimerization which is important for its downstream functions. In coherence with the existing reports, we also demonstrate that B16-F10 cells treated with rIL-6 increased IL-10 release, while berberine inhibited the same. To test whether rIL-6-induced immunosuppressive factors generate tolerogenicity in macrophages, the macrophages were incubated with the supernatant from B16-F10 cells. The native secretion of IL-10 in B16-F10supernatant was analysed by ELISA. The transfer of tolerogenicity in macrophages is indicated by increased IL-10 release. Surprisingly, pre-treatment of macrophages with berberine prevented tolerogenicity transfer even in the presence of high IL-6. We performed docking studies of berberine with STAT-3 protein using Autodock 4. Berberine binds with predicted active site of SH2 domain (586-688) with a binding energy of -6.26 kcal mol. It forms hydrogen bonds with methionine (660) and lysine (658) residues and hydrophobic interactions with tyrosine (640, 657), isoleucine (653, 659), glutamine (644) and leucine (666) residues (Fig.1H). These results indicate that inhibition in STAT3 phosphorylation and dimerization are plausibly because of berberine interacting with its SH2 domain. Collectively, berberine not only attenuates the release of IL-10 from cancer cells but also prevents tolerogenicity transfer to macrophages via inhibition of IL-6-STAT-3-IL-10 axis.

Berberine decreases Arginase-1 expression via STAT-3 inhibition

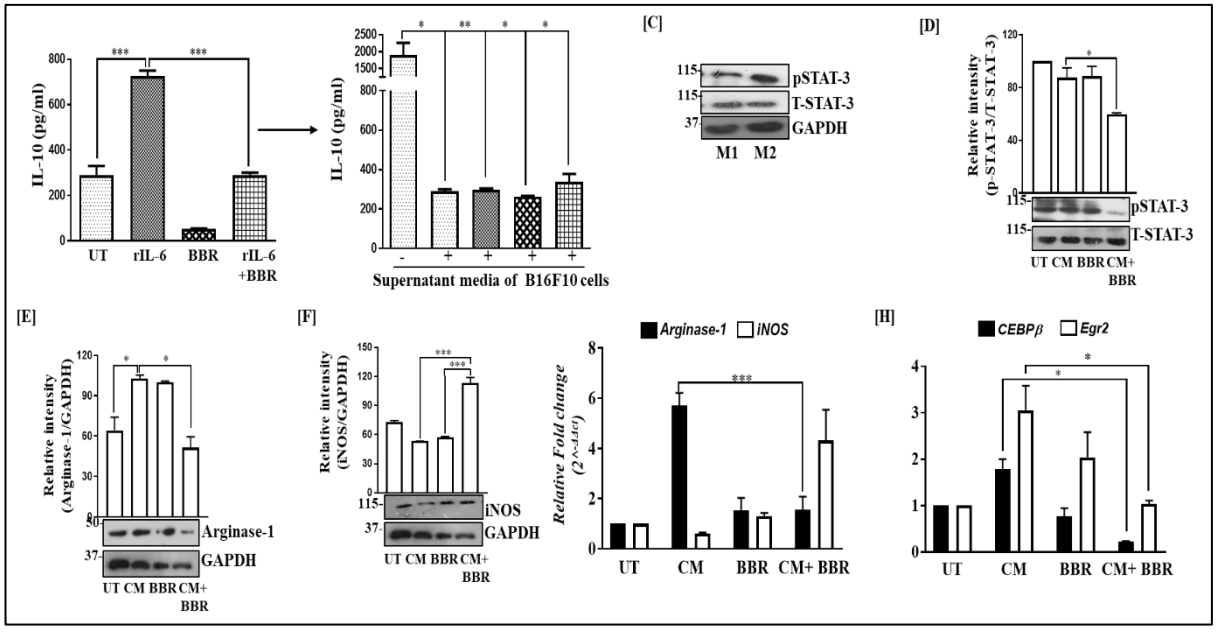


Fig. 18 B16-F10 cells were supplemented with rIL-6 in presence/absence of berberine for 24hrs and IL-10 levels were measured from supernatants. This supernatant was later used to polarise macrophages. BMDM (M0) were pre-treated with berberine and cultured in presence of respective supernatants from the above experiment. IL-10 levels secreted from macrophages were analysed by ELISA to assess tolerogenicity [A and B]. Macrophages grown in M1/M2 cocktail were analysed for pSTAT-3 and T-STAT-3 expression by western blotting. GAPDH was used as an internal control [C]. BMDM were incubated in CM for 5 days followed by treatment with berberine for 12hr to analyse pSTAT-3 and T-STAT-3 and 24hrs for iNOS and Arginase-1 proteins by western blotting. GAPDH was used as an internal control [D-F]. BMDM were incubated

in CM for 24 hrs and were treated with berberine for 6hrs and analysed for Arginase-1, iNOS, C/EPBβand Egr2 transcripts through qPCR analysis and plotted as 2-ΔΔCT values

To test whether rIL-6-induced immunosuppressive factors generate tolerogenicity in macrophages, the macrophages were incubated with the supernatant from B16-F10 cells. The native secretion of IL-10 in B16-F10 supernatant was analysed by ELISA. The transfer of tolerogenicity in macrophages was indicated by increased IL-10 release. Surprisingly, pre-treatment of macrophages with berberine prevented tolerogenicity transfer even in the presence of high IL-6. The immunosuppressive effects of macrophages have been attributed to STAT signalling, importantly STAT-3. STAT-3 phosphorylation has been reported to be associated with Arginase-1 expression (TAM/M2 marker). We observed that the CM-induced macrophages showed high pSTAT-3 and downstream arginase-1 expression with low iNOS. However, berberine skewed the CM-induced macrophages to low arginase-1 and high iNOS expressing subset via STAT-3 inhibition indicating the reversal to M1 macrophages.

Berberine along with L-arginine enhances NO release from CM-induced macrophages

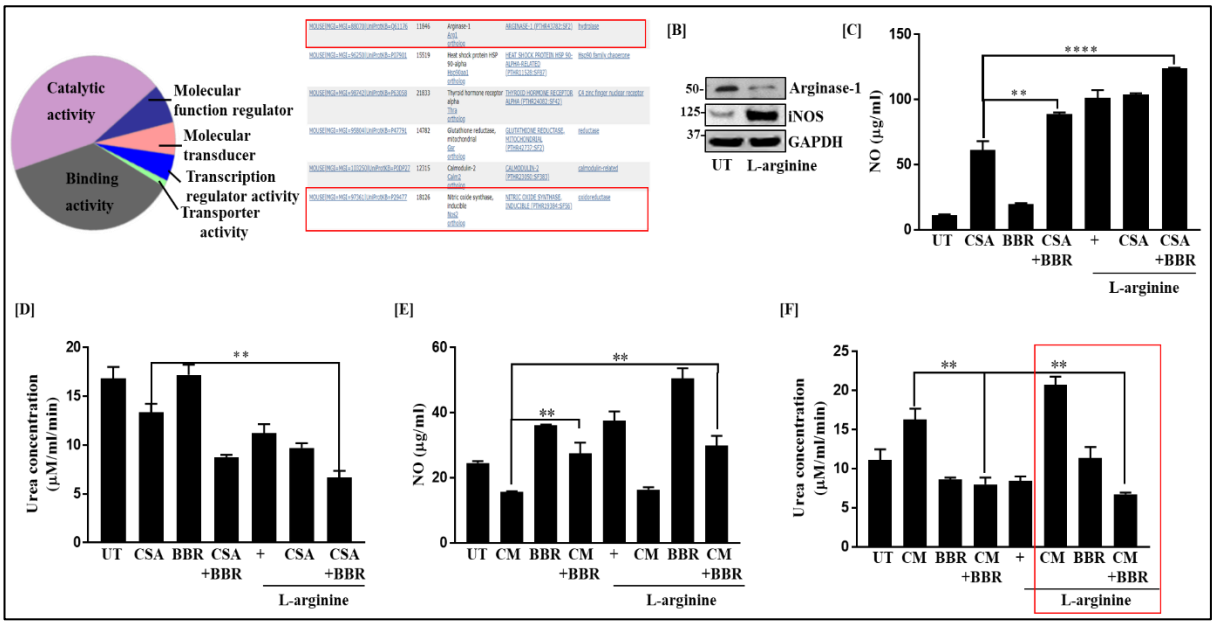


Fig. 19 Upregulation/downregulation of genes involved in functional output by berberine were analysed using Panther [A]. The BMDM were treated with L-arginine for 24 hrs and expression of iNOS and Arginase-1 were checked using western blotting [B]. The BMDM were treated with pre-treated CSA for 12 hours followed by media change. Later, the cells were treated with berberine in the presence/absence of L-arginine for 18 hrs and analysed for NO and urea concentration using Griess reagent and Arginase-1 activity respectively [C and D]. The BMDM were incubated in CM for 4 days followed by berberine and L-arginine treatment for 24 hrs after media change and analysed for NO and urea concentration using Griess reagent and Arginase-1 activity respectively [E and F].

We wanted to check the effect of berberine on L-arginine metabolism. The *in silico* analysis indicated that berberine affects the catalytic activity of iNOS and Arginase-1 proteins. It is reported that supplementing L-arginine enhances iNOS expression in macrophages. So we wanted to check if berberine synergizes with L-arginine supplementation to enhance the iNOS expression. We observed that the macrophages pre-treated with CSA when treated with berberine in the presence of L-arginine showed enhanced NO release and low urea concentration indicating the upregulation of iNOS. Later the CM induced macrophages when treated with L-arginine showed enhanced urea release stating thata the CM-induced M2 macrophages consume L-arginine, enhancing the Arginase-1 activity in TME, while in the presence of berberine, a decrease in urea concentration was observed. This data aligns with our previous observation of berberine polarising macrophages to M1 phenotype and improving NO release even in the presence of L-arginine supplementation.

Exploring the anticancer potential of Berberine in Tumor Microenvironment

Berberine along with L-arginine preferentially skewed CM-induced macrophages to iNOS positive macrophages

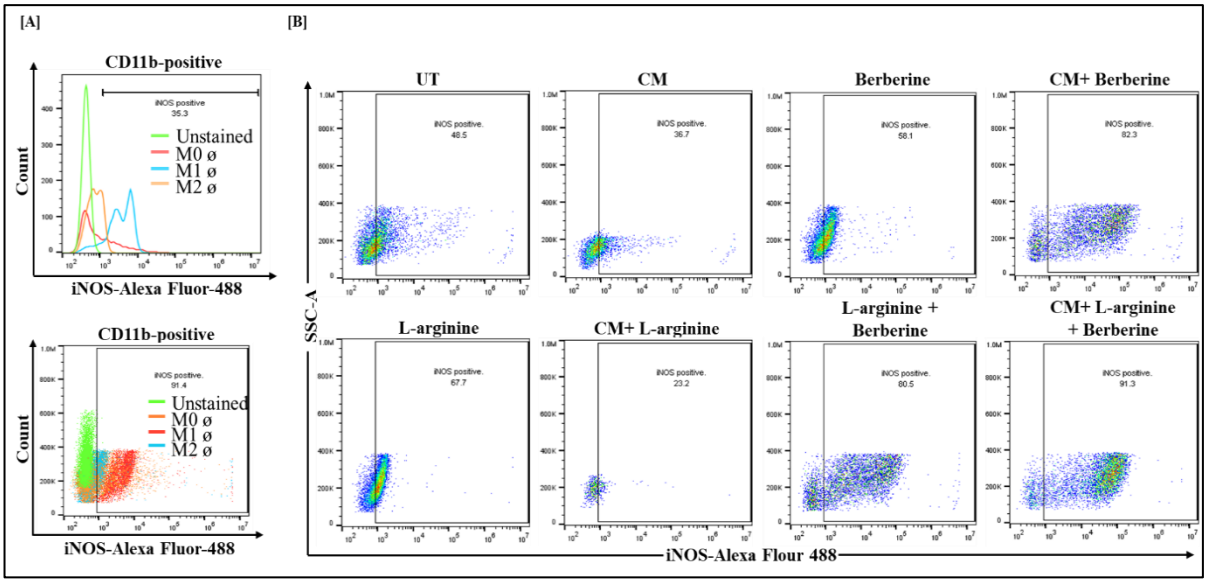


Fig. 20 BMDM were incubated in CM for 4 days followed by berberine and L-arginine treatment for 24 hrs. Later the collected cells were stained for CD11b-APC and iNOS-Alexa Fluor 488 and analysed using flow cytometry. The BMDM were polarized to M1 and M2 macrophages and stained for CD11b-APC and iNOS-Alexa Fluor 488. The cells were gated using FSC vs SSC and CD11b+ cells were gated for iNOS+ cell population and plotted as dot plot [A and B].

We wanted to check if L-arginine supplementation enhances either iNOS or arginase-1 in the presence of berberine. The macrophages were pre-treated with CM followed by treatment with berberine and L-arginine. We observed that treatment with CM stimulated macrophages to release urea and decrease NO concentration. Supplementing L-arginine enhanced iNOS positive macrophages from naïve while there was a decrease in iNOS positive macrophages in CM-induced macrophages even in the presence of L-arginine. Berberine enhanced iNOS positive macrophages in the CM treatment. It also enhanced the frequency of iNOS positive macrophages in the presence of L-arginine in CM-induced *in vitro* TME. This result indicates that berberine preferentially drives the macrophages to iNOS positive which was potentiated in the presence of L-arginine.

Combination of L-arginine and berberine enhances anti-tumor macrophage subset

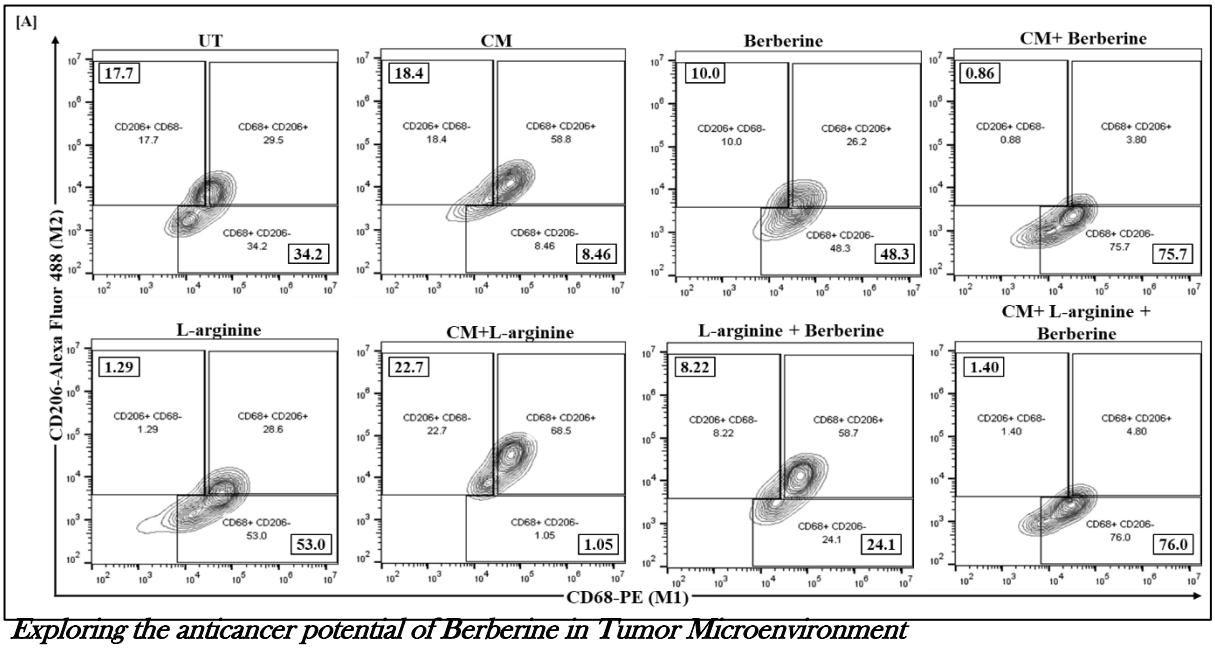


Fig. 21 BMDM were incubated in CM for 4 days followed by berberine and L-arginine treatment for 24 hrs to check for macrophage polarization. Later the collected cells were stained for CD11b-APC, CD206-Alexa Fluor 488 (M2 marker) and CD68-PE (M1 marker), analysed using flow cytometry. The BMDM were gated using FSC vs SSC and CD11b+ cells were further gated for CD206 and CD68 positive cell population [A].

In order to confirm the previous results, we wanted to check if the macrophages were polarizing to M1 phenotype in the presence of berberine and L-arginine treatment. So, CM-induced macrophages were treated with L-arginine and berberine. We have observed that treatment with L-arginine in untreated macrophages skewed macrophages to M1 phenotype but L-arginine supplementation failed to skew macrophages to classical phenotype in the presence of conditioned media. Berberine was able to revert the generated immunosuppressive phenotype to anti-tumor phenotype, which was potentiated by L-arginine. Thus, an increase in iNOS expression in berberine and L-arginine treatment is co-related with skewing of macrophages to anti-tumor phenotype.

Berberine along with L-arginine supplementation decreases release of immunosuppressive cytokines.

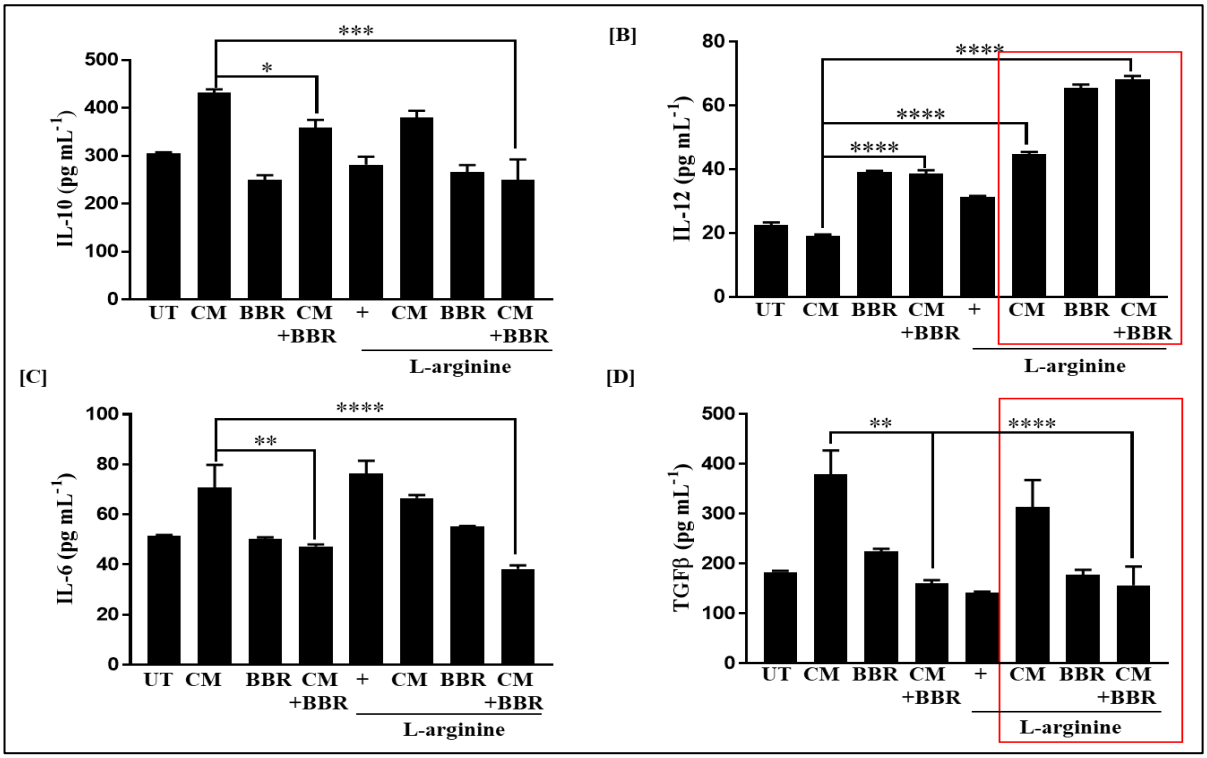


*Fig.22 BMDM were incubated in CM for 4 days followed by berberine and L-arginine treatment for 24 hrs and the cell free supernatant was collected to analyse various immunosuppressive and T cell cytokines [A-D] Data are of three experiments and represented as mean ± SEM. Statistical significance was determined by One-way ANOVA with Turkey's multiple comparison (***p < 0.0001; **p < 0.001; *p < 0.05).*

Later, we polarized the macrophages to M1 and M2 phenotype and checked for various cytokines. We observed that M2 polarized macrophages in presence of TMC showed high arginase-1 activity, which was improved on L-arginine supplementation, but significantly reduced by berberine. In M1 polarized macrophages, extracellular L-arginine enhances NO release by increasing iNOS expression. CM treatment skews M1 macrophages to high arginase-1 expressing phenotype, which was maintained in the presence of L-arginine substrate. It was further confirmed increased release of immunosuppressive cytokines from L-arginine treated CM induced macrophages which was abrogated in the presence of berberine. Even an increase in IL-12 levels was observed from CM macrophages in the presence of berberine and L-arginine. Similar results were observed in polarised macrophages

Berberine in presence of L-arginine reduces mRNA expression of Egr2 and CEBPβ

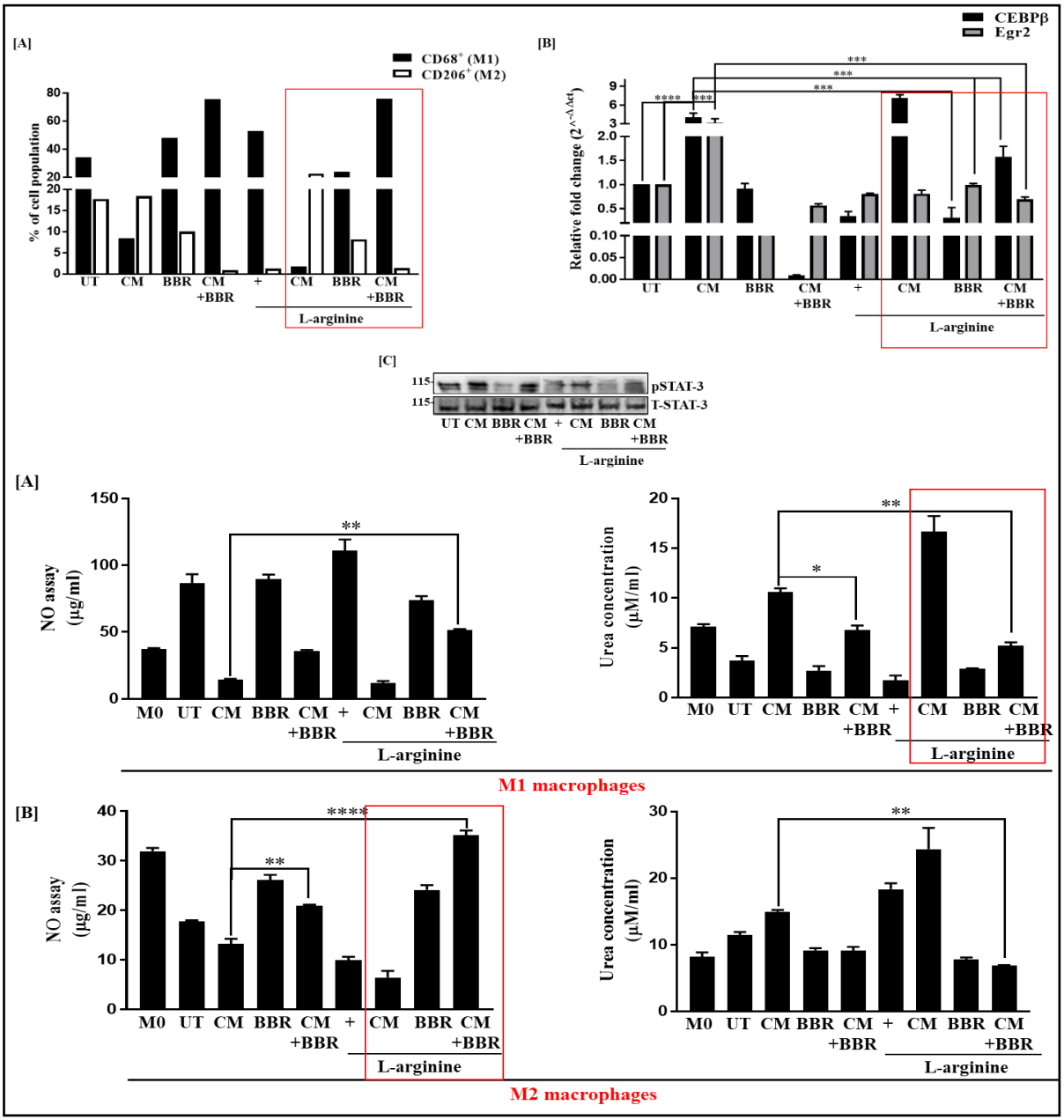


*Fig. 23 BMDM were incubated in CM for 4 days followed by berberine and L-arginine treatment for 24 hrs to check for macrophage polarization. Later the collected cells were stained for CD11b-APC, CD206-Alexa Fluor 488 (M2 marker) and CD68-PE (M1 marker), analysed using flow cytometry. The BMDM were gated using FSC vs SSC and CD11b+ cells were further gated for CD206 and CD68 positive cell population and plotted in graphical representation [A]. BMDM were incubated with CM for 4 days. Later the cells were treated with berberine and L-arginine for 6 hrs and analysed for CEBPβ and Egr2 transcripts through qPCR and plotted as 2-ΔΔCT values [B]. The BMDM were incubated with CM for 4 days followed by berberine and L-arginine treatment for 3 hrs and analysed for pSTAT-3 and T-STAT-3 by western blotting [C]. BMDM were polarised to M1 and M2 macrophages using the cocktail of GMCSF+ IFNγ/ MCSF+ IL-4+IL-6 respectively. Later the polarised macrophages were incubated in CM for 2 days followed by berberine and L-arginine treatment for 24 hrs and analysed for NO release and urea concentration using Griess method and Arginase-1 activity [A and B]. Data are of three experiments and represented as mean ± SEM. Statistical significance was determined by One-way ANOVA with Turkey's multiple comparison (***p < 0.0001; **p < 0.001; *p < 0.05).*

To elucidate the molecular mechanism of action for skewing of macrophages to M1-phenotype in the presence of berberine and L-arginine, we checked for STAT-3 mediated up regulation of CEPBβ and Egr2. We have observed that CM-induced macrophages had high expression of CEBPβ and Egr2 which

was maintained in the presence of L-arginine. While a decrease was observed in mRNA transcript expression of CEBPβ and Egr2 in the presence of berberine and L-arginine. So, the overall data indicates that combination of berberine along with L-arginine downregulates STAT-3 expression which in turn decreases CEBPβ and Egr2, skewing the CM-induced M2 macrophages to high iNOS positive M1 macrophages. It was even confirmed in the presence of polarized macrophages.

Berberine and L-arginine enhances the secretion of T cell priming cytokine (IL-12)

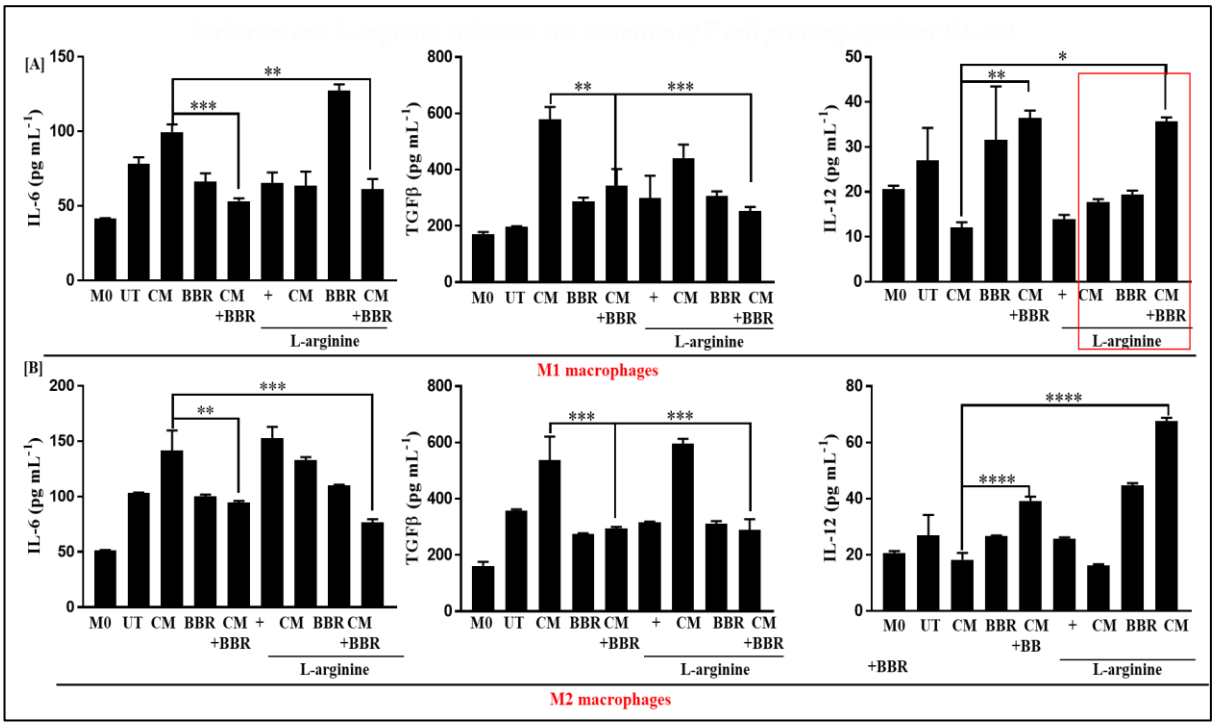
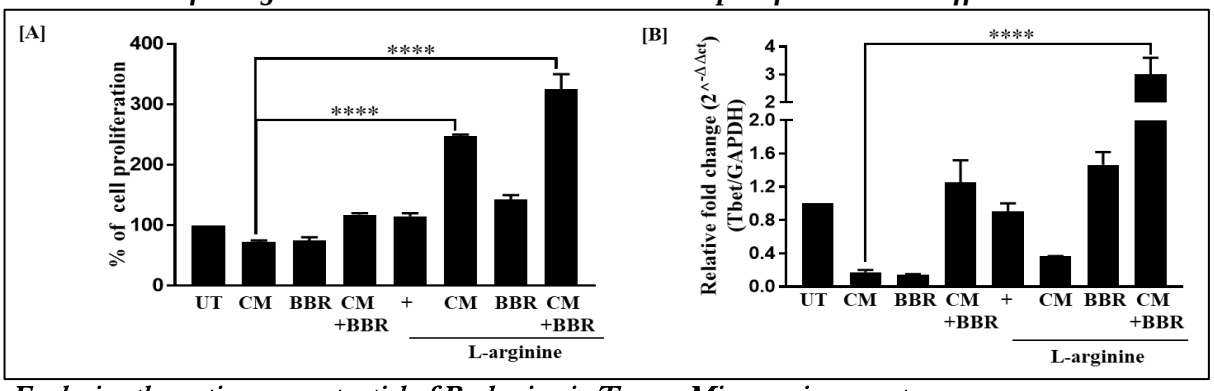


*Fig. 24 BMDM were polarised to M1 and M2 macrophages using the cocktail of GMCSF+ IFNγ/ MCSF+ IL-4+IL-6 respectively. Later the polarised macrophages were incubated in CM for 2 days followed by berberine and L-arginine treatment for 24 hrs and analysed for various immunosuppressive and T cell activating cytokines by ELISA [A and B]. Data are of three experiments and represented as mean ± SEM. Statistical significance was determined by One-way ANOVA with Turkey's multiple comparison (***p < 0.0001; **p < 0.001; *p < 0.05).*

The previous results were further confirmed that berberine along with L-arginine skew macrophages to M1 phenotype. To further confirm the functional aspect of macrophages, the polarised macrophages were treated with CM and berberine in the presence/absence of L-arginine. We have observed that berberine along with L-arginine enhanced IL-12, which is a T cell priming cytokine. On contrary, it we observed a decrease in immunosuppressive cytokines IL-6 and TGFβ on polarised macrophages. So, these results indicate that combination of berberine and L-arginine not only decreases immunosuppressive cytokines (IL-6 and TGFβ), while it helps in upregulation of IL-12 in CM-induced polarised macrophages.

Combination of L-arginine and berberine enhances T cell proliferation and differentiation



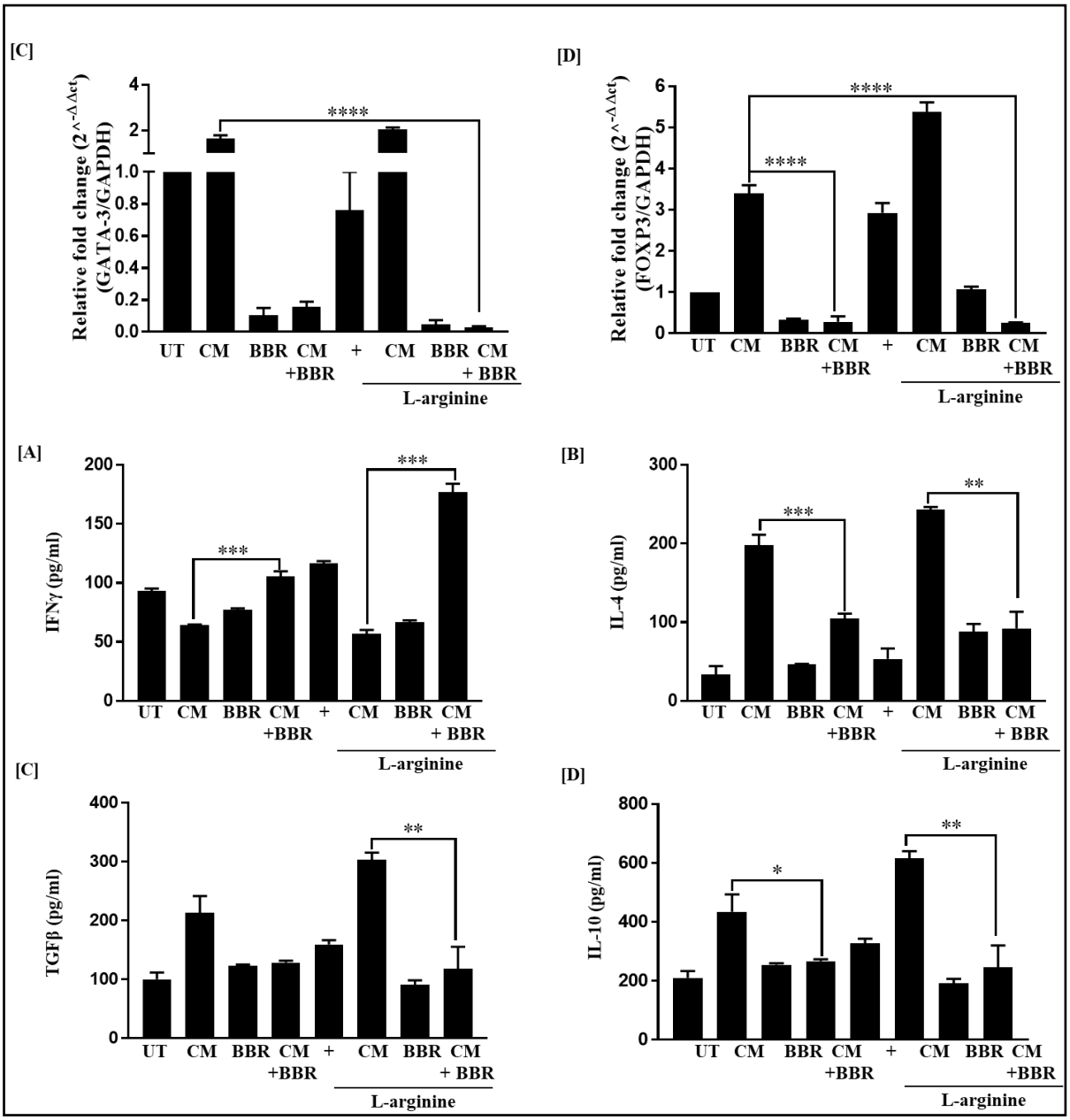


*Fig. 25 The splenocytes were cultured in the CM media in the presence/absence of berberine and L-arginine and cellular proliferation was checked by MTT assay [A]. BMDM were incubated in CM for 4 days followed by treatment with berberine and L-arginine for 24 hrs. Later, total T cells were purified using Nylon wool and co-cultured for 12 hrs. The cells were collected in TRIzol for RNA isolation. The cDNA was prepared and used for analysis for T cell transcription factors qPCR and plotted as 2-ΔΔCT values [B-D]. . BMDM were incubated in CM for 5 days followed by treatment with berberine and L-arginine for 24 hrs. Later, CD4+ T cells were purified co-cultured for 48 hrs and collected supernatant was used to analyse various cytokines by ELISA [A-D]. Data are of three experiments and represented as mean ± SEM. Statistical significance was determined by One-way ANOVA with Turkey's multiple comparison (***p < 0.0001; **p < 0.001; *p < 0.05).*

Extrapolating the above results to adaptive immune response, we have observed an increased proliferation of CD4 T cells in combination treatment. The CD4 T cells were differentiated to Th1 phenotype as evident by high T bet and low GATA-3 and FOXP3 mRNA expression. It was further confirmed by increase in IFNγ release and decrease in IL-4, IL-10 and TGFβ. These results indicate that the combination of L-arginine and berberine enhances innate as well as adaptive immunity. Thus, it may serve as a potential anticancer therapeutic.

Combination of L-norvaline and berberine enhances iNOS expression in CM primed macrophages

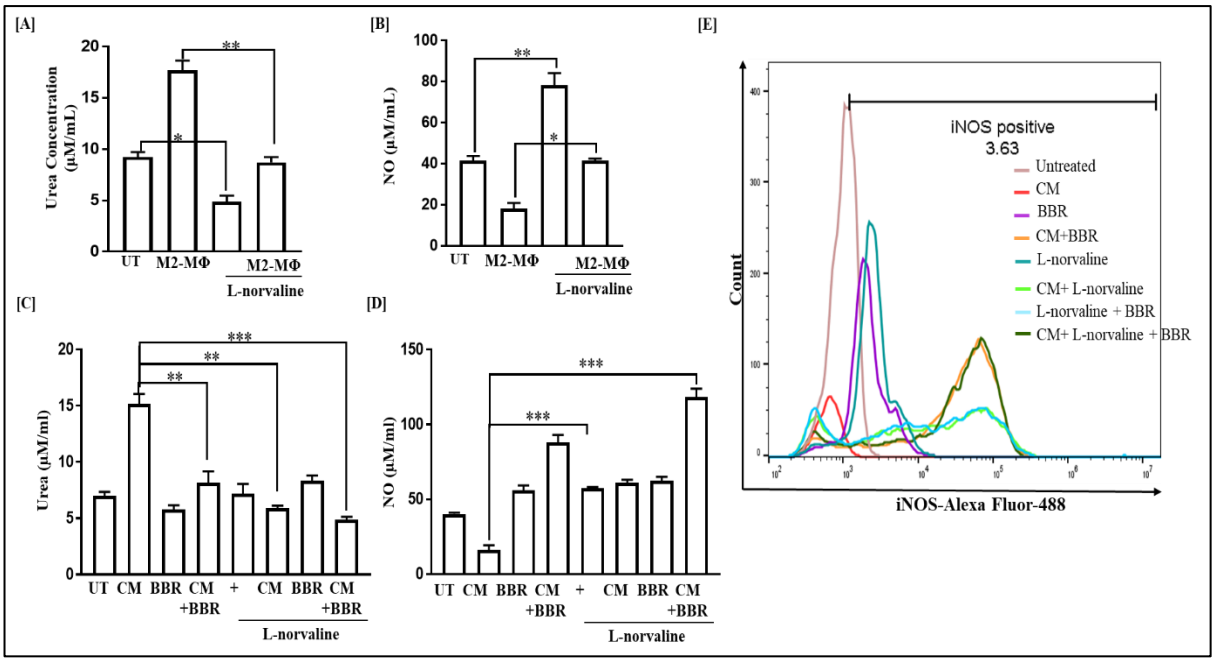


Fig. 26 The M2 polarized macrophages were treated with L-norvaline and checked for urea release by Arginase-1 activity [A]; NO release by Griess assay [B]. BMDM were incubated in CM for 4 days followed by treatment with berberine and L-norvaline for 24 and analysed for urea by Arginase-1 activity [C]; NO release by Griess reagent [D]. The CM primed BMDM were treated with berberine and L-norvaline for 24 hrs. The collected cells were stained for CD11b-APC and iNOS-Alexa Flour 488 and analysed using flow cytometry. The BMDM were gated using FSC vs SSC and CD11b+ cells were further gated for iNOS expression and plotted as histogram [E].

Previous results indicated that supplementing L-arginine substrate either enhance Arginase-1 or iNOS enzyme depending on the macrophage phenotype, so we wanted to check the effect of berberine in presence of Arginase-1 inhibitor (L-norvaline). As observed from above results, CM induced macrophages show high Arginase-1 activity. In order to check the effect of L-norvaline on CM-induced immunosuppressive macrophages, the CM-induced macrophages were treated with L-norvaline for 24 hrs and checked for urea concentration. We observed that CM induced macrophages showed increased release of urea concentration, which was abrogated in the presence of L-norvaline. To confirm the results, we incubated the cells in CM for 12 hrs. Later, the cells were treated with L-norvaline and berberine for 24 hrs, and analysed for Arginase-1 activity and NO assay. We observed that CM induced macrophages showed high Arginase-1 activity and low NO release, while berberine reduced urea release and enhanced NO from CM-induced macrophages. The concentration of urea is reduced significantly by L-norvaline in CM treated macrophages. Simultaneously, there was an increase in the NO release in the presence of L-norvaline indicating that intracellular arginine was consumed by iNOS to produce NO. The treatment with berberine in TMC induced macrophages showed decreased arginase-1 activity while increased release of NO. We observed a decrease in the arginase-1activity in the presence of berberine and L-norvaline indicating a synergy between L-norvaline and berberine, which was further reduced in the CM-induced macrophages. It was further confirmed by increase in the iNOS expression from macrophages. Thus, the data indicates that berberine synergizes with Arginase-1 inhibitor (L-norvaline) to decrease arginase-1 activity.

L-norvline along with berberine reduces tumor growth in vivo

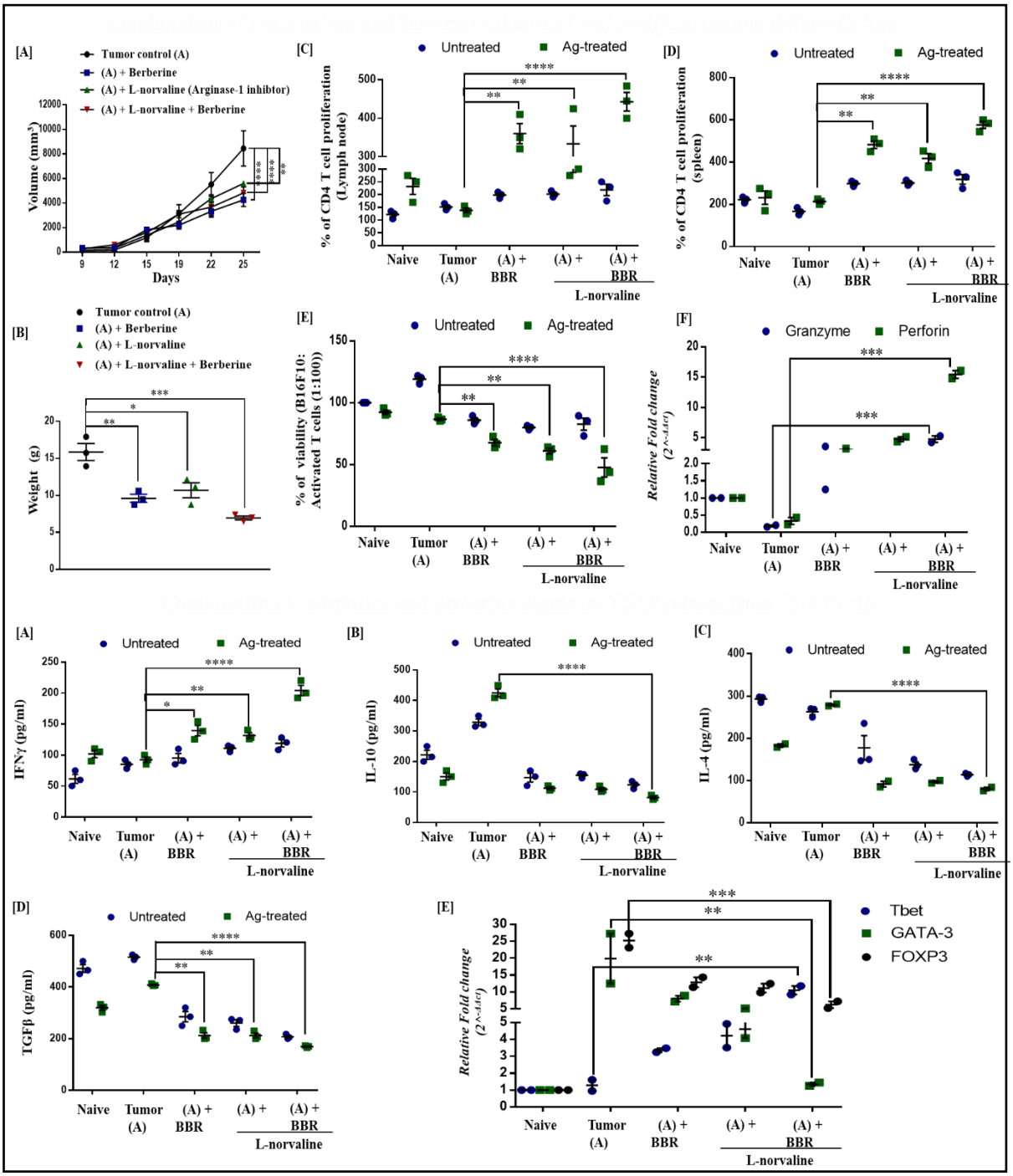


Fig. 27 The tumor bearing mice were treated with L-norvaline, berberine and in combination and the excised tumor tissue was analysed for weight and volume [A and B]. The BMDM were primed CSA for 24 hrs, co-cultured with the CD4+ T cells and checked for proliferation MTT assay [C and D] and CTL assay [E]. RNA was isolated from the lymph node of tumor control and treatment group and analysed for key Granzyme and perforin by qPCR normalized to β actin and plotted as 2-ΔΔCT values [F]. The BMDM were primed with crude soluble antigen for 24 hrs and later co-cultured with the CD4+ T cells, isolated from the lymph nodes of tumor control and treatment groups for 72 hrs and supernatant was used to score various cytokines by ELISA [A-D] . RNA was isolated from the lymph node of tumor control and treatment group and analysed for key transcription factors by qPCR normalized to β actin and plotted as 2-ΔΔCT values [E]

In order to check the efficacy of berberine and Arginase-1 inhibitor in vivo, B16-F10 cells were injected subcutaneously into the right flank of C57BL/6 mice. After the tumor was palpable, L-norvaline

(Arginase-1 inhibitor) and berberine were intraperitoneally injected on alternate days. The weight and volume of tumor from different treatment groups were measured. We observed a reduction in tumor volume and weight in both berberine and L-norvaline individually as well as in combination treatment. The spleen volume was increased in the tumor bearing mice which was reduced in the treatment groups. Later, we wanted to check T cell proliferation from the tumor bearing treatment groups, so we re-challenged the bone-marrow derived macrophages with tumor antigen and co-cultured with CD4 T cells isolated from the lymph node of the tumor bearing groups. We observed that T cell proliferation in the tumor group was significantly low which was rescued by berberine or L-norvaline treatment or combination treatment. The combination treatment showed better efficacy than berberine or L-norvaline alone. This was even supported by release of low IFNγ and high IL-10 from tumor group which was rescued in treatment groups. Increased release of IFNγ and low IL-10 from treatment groups may indicate high CD8 or Th-1 Tcells in the tumor microenvironment.

Discussion:

With recent understanding regarding the importance of immune cells in TME, developing a novel therapeutics targeting the immune cells in the microenvironment would be a better strategy. Since most of the drugs targeting molecular targets have failed in clinical trials, we aimed at testing the effect of berberine on macrophage polarization and subsequent immune alterations. STITCH analysis, which curate protein-chemical interactions showed that berberine may interact with iNOS and STAT-3 (SF-6A). Earlier reports have indicated the importance of these proteins in polarized macrophages22. Berberine has been explored for immuno-modulatory effects in various infection model and chronic diseases like diabetes, but still its molecular mechanism of action in the TME needs to be studied in detail. The manuscript discusses the effect of berberine in the neuroblastoma-induced immunosuppressive microenvironment by demonstrating that (i) it prevents the transfer of IL-6-STAT-3-IL-10 mediated tolerogenicity to macrophages, (ii) reverts an immunosuppressive microenvironment to a pro-inflammatory environment, (iii) it causes a phenotypic and functional skewing of CM-induced or M2 subset to anti-tumor M1 subset, (iv) it primes a TH-1 response even at the CM via improving the expression of MHC-II and CD40 and facilitates the mounting of CTL response (v) it reduces the tumor growth and subsequently improves tumor infiltrating M1 population in vivo

STAT-3 is a ubiquitous signaling molecule present in most cells depending on developmental stage and origin. It is indicated to contribute to oncogenesis. In TME, the order of its expression is as cancer cells>> TAMs>> inflammatory macrophages. Thus targeting STAT-3 inhibition in the cancer cells and parallel in the myeloid cells may serve as a better strategy. Various cancer cell survival and progression molecules such as MCSF, IL-6, IL-10 etc are regulated via STAT-3 phosphorylation. It has feed-forward loop which strongly operates between cancer-immune cells through autocrine/paracrine manner. In the B16F10 cells, we demonstrate that berberine inhibits pSTAT3 in dose and time dependent manner by plausibly interacting with the SH2 domain of the STAT-3 protein (Fig.1A-E). It also attenuates IL-6 induced IL-10 release that serves as tumor-promoting loop in cancer. The conditioned media of B16F10 cells induced tolerogenicity in the macrophages. However, the macrophages pre-treated with berberine demonstrated a significant reduction in IL-10 release. IL-10 is secreted by various cells such as cancer cells, TAMs, TH-2 and regulatory T-cells at TME. It has been reported to decrease antigen presentation and generates pro-tumor macrophage.

Soluble factors secreted from cancer cells skews macrophage towards alternative phenotype (M2). Tumor induced immune suppression is better replicated with cancer cell antigens and secretome (CM). Hence, CM cultured macrophages differentiated to a subset that showed MHC-IIlow and CD206high indicating the generation of TAM-like M2 phenotype which was confirmed by TGF-β and IL-10 release. TAMs employ various mechanism like T-cell suppression and anergy, enhancing angiogenesis,

metastasis, secretion of immunosuppressive cytokines etc leading to tumor growth8. One of the immunosuppressive cytokine released by TAMs is IL-6. IL-6 in TME functions as anti-/pro tumorigenic cytokine depending on the macrophage phenotype. This cytokine, if secreted in the presence of IFNγ induces the release of pro-inflammatory cytokines while it drives macrophages towards M2 in the presence of IL-4. In TME, IL-6 secreted from macrophage helps in maintenance of the immunosuppressive environment and aids in cancer progression. We observed that berberine reduces the release of IL-6 in the rIL-10 induced immunosuppressive environment. Along with that, it mediated decrease in the CM-induced TAM-like M2 phenotype which is evident from decreased MHC-II-CD206+ population. Moreover, String analysis with the berberine manipulated proteins and mRNA in the studied revealed a network. On analysing the network with ontology and scoring in cytoscape, IL-6, IL-10, TGFβ and, MHC-II might be manipulated not only through STAT-3 but multiple pathways manipulated by berberine converging to them thus enhancing the effect (Fig. 7). A master regulator of macrophage polarisation is the transcription factor C/EBPβ. C/EBPβ induces alternative macrophage activation and is highly expressed in TAMs. Early Growth Response2 (Egr2), a well-studied M2 marker also induces C/EBPβ transcription by binding to its promoter region. IL-6 mediated STAT-3 also induces C/EBPβ mediated changes. In our study, berberine abrogated Egr2 and C/EBPβ expression in CM-treated macrophages indicating that berberine modulates upstream signals mandatory for M2 associated gene expression. It is concurrence with our observation that berberine inhibits STAT-3 thus inhibiting the M2 generating lineage of signals including Erg2 and C/EBPβ.

Berberine is reported for its anti-inflammatory properties in infection models of LPS or autoimmune diseases. However, similar to our results, F Zhu et al, have reported that berberine elevates IL-1β levels in Alzheimer's disease; while Saha et al observed increased iNOS and nitric oxide levels along with a downregulation of IL-10 during leishmanial infection; T S kim et al., reported that berberine increased IL-12 secretion from macrophages stimulated with LPS; Berberine enhanced TNFα production from cancer cells . It also sensitizes cancer cells to TNFα treatment. These reports indicate that berberine may display varied pro/anti-inflammatory response depending on the nature and quantity of antigen, its working concentration etc. In consistence with the above observation, our results showed that berberine induced pro-inflammatory response and the effect was much more pronounced in the immunosuppressive micro-environment. Moreover, berberine reduces the melanoma tumor growth. It enhances the population of pro-inflammatory macrophages (F4/80-iNOS double positive, CD68 expression) and also reduces the protein expression of CD206, pSTAT-3 and Arginase-1 in the TME in coherence with the in vitro results.

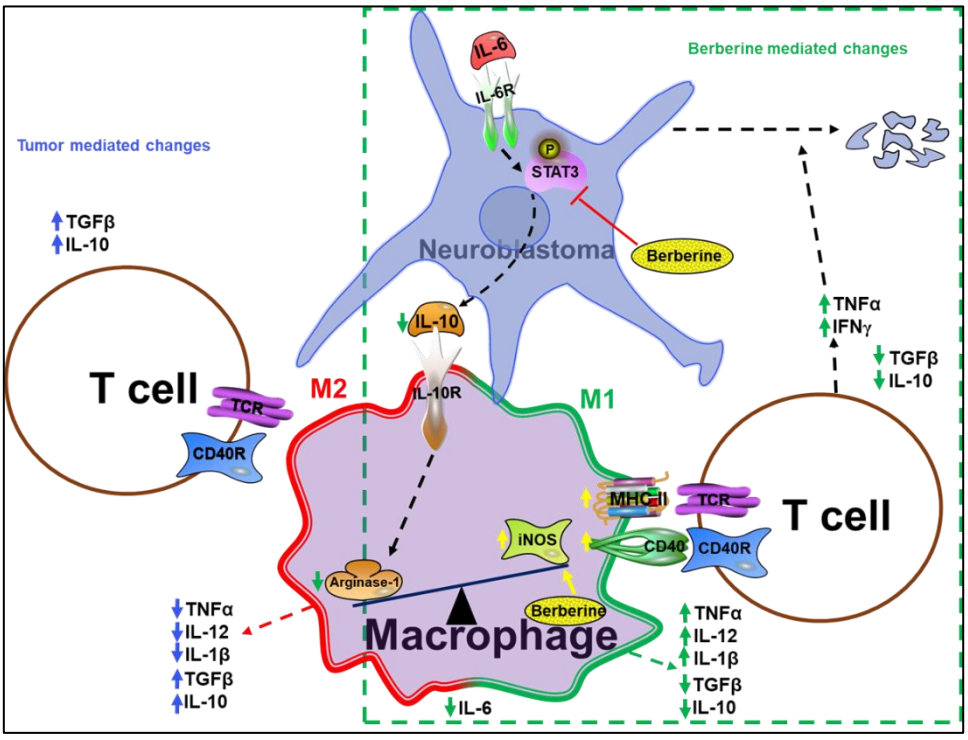
For an effective immune response against cancer cells, macrophages should efficiently prime T-cells to mount an anti-tumor immune response, however TME primes the T-cells to regulatory T-cells causing a failure in mounting an appropriate immune response. We demonstrated that berberine enhanced MHC-II and co-stimulatory molecule CD40 expression that is reported to be downregulated during cancer. CD40 expression is attributed as major reasons for failure in mounting an appropriate anti-tumor T-cell response5. CM primed macrophages co-cultured with T-cells induced FOXP3 and GATA-3 transcription confirming the previous observations regarding the induction of regulatory T-cells or TH-2 immune response and failed to induce T-bet transcription in T-cells which has been reported. Surprisingly berberine decreased FOXP3 and GATA-3 transcription enhancing the T-bet transcription and CTL activity with increased IFNγ which improves T-cell efficacy.

In concurrence with the existing reports and ongoing work, our results suggest that berberine modulates tumor microenvironment and reverts the skewing of TAM-like M2 macrophages to activated M1 macrophage and in turn activates the CD4+ T-cell response and CTL activity. Moreover, these results have been extrapolated to *in vivo* model of melanoma indicating a promising efficacy of berberine in restoring the inflammatory macrophages in TME.

Extracellular L-arginine supplementation aids in enhancing immunity. Reports have reported that L-arginine supplementation in immune cells enhances their proliferation and survival. In macrophages, extracellular L-arginine drives macrophages to anti-cancer phenotype. It is reported that L-arginine supplementation upregulates iNOS transcripts widely known as L-arginine paradox. But in the tumor microenvironment, there is an accumulation of tumor associated macrophages. TAMs consume extracellular L-arginine by Arginase-1 to secrete urea and polyamines, which in turn favours tumor growth and survival. In our study, we have observed that L-arginine supplementation in CM-induced macrophages showed high urea release compared to untreated macrophages but this was abrogated in the presence of berberine. So in order to determine a therapeutic regime for tumor microenvironment we have used a combination of L-norvaline and berberine to combat tumor growth. We have observed that berberine along with L-norvaline showed significant decrease in tumor growth and survival. Hence, with this study, we have tried to answer the molecular mechanism of berberine on skewing the macrophages to anti-tumor phenotype via targeting L-arginine metabolism.

Conclusion:

The presented work indicates that berberine aids in restoration of M1 macrophages in TME. It also enhances CD40 expression which play an important role in activation of adaptive immune response which was confirmed by increased CD4 T cell proliferation and CTL activity. The results were extrapolated in *in vivo* model of solid tumor. Berberine treatment group showed significantly decrease in tumor growth. Apart from that, we have observed that combination of L-norvaline (Arginase-1 inhibitor) and berberine may serve as a better therapeutic strategy to combat tumor.



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- Challagundla N*, Shah D*, Yadav S, Agrawal-Rajput R. Saga of monokines in shaping tumour-immune microenvironment: Origin to execution. Cytokine. 2022 Sep1;157:155948 (* equal contribution).

- Shah D, Challagundla N, Dave V, Patidar A, Saha B, Nivsarkar M, Trivedi VB, Agrawal-Rajput R. Berberine mediates tumor cell death by skewing tumor-associated immunosuppressive macrophages to inflammatory macrophages. Phytomedicine. 2022 May 1;99:153904

- Shah D, Yadav S, Shah J, Palkhade R and Agrawal-Rajput R. Berberine synergizes with L-norvaline (Arginase-1 inhibitor) to reduce tumor growth (manuscript under preparation)

- Patel D, Challagundla N, Mandaliya D, Yadav S, Naik O, Dalai P, Shah D, Vora H, Agrawal-Rajput R. Caspase-1 inhibition by YVAD generates tregs pivoting IL-17 to IL-22 response in β-glucan induced airway inflammation. Immunopharmacology and Immunotoxicology. 2022 May 4;44(3):316-25

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- Dalai P, Shah D, Thanki K, Shah J and Agrawal-Rajput R. Doxycycline along with CD39 and CD73 manipulates ATP metabolism to mediate anti-cancer immunity (manuscript under preparation)

- Challagundla N, Shah D, Dalai S, Agrawal-Rajput R. IFNγ insufficiency during mouse intravaginal Chlamydia trachomatis infection exacerbates alternative activation in macrophages with compromised CD40 functions. European Journal of Immunology. (Submission ID: eji.202250064)

Conferences:

- Presented a Presentation for Dr. G.P. Talwar Young Scientist Award presentation: Dhruvi Shah and Reena Agrawal-Rajput "Berberine aids in skewing immunosuppressive macrophages to anti-tumor phenotype in TME via modulating L-arginine metabolism" at 48th Annual Meeting of Indian Immunological Society, 8th-9th July 2022 held at BHU, Varanasi
- Presented a Poster: "Berberine restores inflammatory macrophage and subsequent T cell activation in Tumor micro-environment" at 46th Annual Meeting of Indian Immunological Society in Advances in Immunology Research: Impact on Healthcare, 14th-16th Nov 2019 held at BARC, Mumbai
- Presented a Poster: "Berberine rescues Macrophage Polarization altered by the Tumor Micro-environment" at 45th Annual Meeting of the Indian Immunology Society conference, 1st-3rd Nov, 2018 held at THSTI, New Delhi
- Awarded Best Poster Presentation: "Targeting the immunosuppressive tumor microenvironment with Berberine" at National Conference on New Horizons in Cancer Biology organized by The Gujarat Cancer & Research Institute, 16-17th March 2018
- Presented a Poster "Exploring the effect of Berberine on Neuroblastoma for its Anti-tumor Immune Response" at 44th Annual Conference of the Indian Immunology Society conference, 14-17th Dec, 2017 held at Nirma University, Ahmedabad**.**